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Vitamin A equivalence of the β-carotene in biofortified cassava in women

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ABSTRACT

Cassava is an important food energy source in tropical and subtropical regions. There have been successful efforts to increase the β-carotene content in cassava through biofortification. However, the bioefficacy of the β-carotene in the biofortified cassava in humans has not been studied. In the present study, we investigated the bioefficacy of the β-carotene in biofortified cassava in 9 healthy women ages 18-39 y. After eating conventional foods with low β-carotene and vitamin A contents for three days, the subjects were asked to ingest one of the three cassava porridges in random order. All of the porridge servings were about 200 g and contained 40 g cassava flour. The three porridges included biofortified cassava porridge (1097.5 μg β-carotene), white cassava porridge with vitamin A reference dose (285.6 μg retinol) and white cassava porridge with β-carotene reference dose (537.6 μg β-carotene). Blood samples were collected prior to ingestion of the test porridge and at 2, 3.5, 5, 6.5, 9 hours after ingestion. Plasma triacylglycerol rich lipoproteins (chylomicrons and large VLDL) were isolated and the retinyl palmitate contents were analyzed by HPLC-ECD. The mean amounts of retinyl palmitate appearing in the triacylglycerol-rich lipoprotein fraction in the entire plasma pool after ingestion of the biofortified cassava porridge and the white cassava porridge with the β-carotene reference dose were 1587.53 ± 285.27 and 914.35 ± 128.50 nmol. The vitamin A equivalence values of the β-carotene in these two porridges were 2.80 ± 1.77 to 1 and 2.11 ± 0.81 to 1 (by weight), respectively. These vitamin A equivalence values were not significantly different by t test. In our study population, the bioefficacy of the β-carotene in biofortified cassava was as good as that of a β-carotene supplement.

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Vitamin A deficiency is a common public health problem in developing countries due to diets deficient in vitamin A. Preschool children and pregnant women are most vulnerable to vitamin A deficiency (World Health Organization 1995). It is estimated that vitamin A deficiency affects 75 to 252 million preschool children each year (Maziya-Dixon et al 2006). Vitamin A deficiency may result in blindness, night blindness, decreased immunity, and increased morbidity and mortality (Sommer et al 2008). To combat vitamin A malnutrition in developing countries, efforts have focused upon providing vitamin A supplements for children and pregnant women and vitamin A fortification. There is strong evidence that vitamin A supplementation programs have reduced mortality among infants and pregnant women (West et al 2003). However, impoverished populations heavily rely on plant sources of provitamin A carotenoids to meet their vitamin A requirements (West et al 2003). Biofortification is the process to develop staple crops containing high micronutrient contents using conventional plant breeding technology and/or modern biotechnology (Nestel et al 2006). Through biofortification, crops will be loaded with high levels of minerals and vitamins in their seeds and roots, which will provide multiple benefits. Staple crops are the predominant food source for many low-income families in developing countries. The strategy of biofortification of regularly consumed staples crops targets every family member in micronutrient-deficient populations (Nestel et al 2006). Biofortification is a cost-effective and sustainable process. Once the biofortified crops are developed, they will be able to be grown and to produce more seeds and roots. The seeds or roots can be shared internationally

to improve the nutritional status of people all over the world, even if the formal investment in biofortification research ends. Current biofortification research efforts include developing genotypes of cassava with higher provitamin A carotenoid contents in the starchy roots. Subsequently, the germplasm of those varieties will be shared within the developing world (HarvestPlus 2006).

Cassava (*Manihot esculanta*) serves as a major energy source for about 500 million people in tropical and subtropical regions, because it grows well under marginal conditions of soil and climate, survives diseases and pest attack and yields more energy per hectare compared with other crops (Montagnac et al 2009). The provitamin A carotenoids found in cassava are primarily β-carotene. However, in white cassava, there may be trace amounts of β-carotene, which may be present in concentrations as low as 1 mg/g fresh weight or 3 mg/g dry weight (Iglesias et al 1997, Chavez et al 2005).

The bioavailability of β-carotene from different plant sources varies considerably and is unpredictable (Reboul et al 2006). Therefore it is essential to experimentally determine the vitamin A equivalence of the β-carotene in biofortified cassava. We introduced a highly sensitive HPLC detection technique, coulometric array electrochemical detection (ECD), to quantify the minute vitamin A concentrations in the postprandial plasma triacylglycerol-rich lipoprotein fractions after subjects ingested a single realistic test meal (White et al, 2008). The objective of the current study is to quantify the vitamin A equivalence of the β-carotene in biofortified cassava based on the ingestion of a single realistic serving of cassava porridge.

Literature review

Vitamin A absorption and transport

Mechanism of vitamin A absorption and transport

Vitamin A can be found in two major forms in both animal and plant foods, as retinyl ester and as provitamin A carotenoids (mostly β-carotene), respectively. Dietary retinyl esters are hydrolyzed into retinol by pancreatic triglyceride lipase and the intestinal brush border enzyme phospholipase B in the intestinal lumen prior to uptake into enterocytes. Carotenoids are absorbed directly by enterocytes at a lower efficiency and converted into retinol afterwards. Retinol is reesterified into retinyl esters and incorporated into chylomicrons before being secreted into the lymph. The retinyl esters are then transported to the liver for further metabolism. Retinyl esters in plasma and liver are predominantly retinyl palmitate and retinyl stearate regardless of the composition of the fatty acids in the diet (Berr and Kern 1984).

The mechanism of the intestinal uptake of carotenoids has been thought to be simple diffusion similar to other dietary lipids. The micelle fractions have a disk-like structure, with an outer shell consisting of bile salts, as well as a core of more hydrophobic lipids, where carotenoids are incorporated. It has not been well understood how carotenoids are transported from the micelle core into the enterocytes. However, both in vitro and in vivo studies have shown that pancreatic phospholipase A_2 (PLA_2) is necessary for the cleavage of phospholipids, which is an important step in carotenoids absorption (Yonekura and Nagao 2007). Phosphatidylcholine (PC) suppresses lutein and β-carotene absorption in a dose-dependent manner (Sugawara et al 2001). When micelle fractions were mixed with

phosphatidylcholine (PC), the absorption of the carotenoids was inhibited in rats, as shown by β-carotene responses in the plasma and liver (Baskaran et al 2003) and Caco-2 cells, as shown by micellar β-carotene and lutein levels (Sugawara et al 2001). However the absorption was restored by PLA_2, which indicated the importance role that PLA_2 plays in carotenoid absorption (Sugawara et al 2001, Homan et al 1998).

The simple diffusion mechanism of absorption has been supported by studies which showed a linear response to increasing carotenoid concentrations in perfused rat intestines (Hollander and Ruble 1978) and in rat small intestinal cells (Scita et al 1992). However, recent studies revealed that a receptor-mediated transport also exists (Yonekura and Nagao 2007). There is a pathway for β-carotene and lutein transport across the apical membrane of enterocytes, which involves the scavenger receptor class B type I (SR-BI) (Yonekura and Nagao 2007). Evidence supporting this theory was first found in *Drosophila*. In this species, a gene encoding an SR-BI-homologous protein was shown to be important for carotenoid uptake (Kiefer et al 2002). In SR-BI knockout mice, β-carotene intestinal absorption was significantly reduced, which indicated that β-carotene absorption was partly mediated by SR-BI. The absorption and transport of β-carotene (During 2005) and lutein (Reboul et al 2005) was partly mediated by SR-BI as well in Caco-2 cells, because the anti-SR-BI antibody largely impaired the absorption of β-carotene.

Bioavailability and bioconversion of carotenoids

There are several terms to describe the efficiency of carotenoid absorption and utilization, including bioaccessibility, bioavailability, bioconversion, and bioefficacy. Bioaccessibility refers to the proportion of a carotenoid that is transferred from the food matrix to micelles during digestion and made accessible for intestinal absorption (Stahl 2002).

Bioavailability is the proportion of an ingested carotenoid that can be utilized for normal physiological function or storage (Castenmiller and West 1998). Bioconversion means the fraction of bioavailable provitamin A that can be converted into retinol (Castenmiller and West 1998). Bioefficacy refers to the efficiency of the process that ingested dietary provitamin A carotenoids are absorbed and converted to retinol (van Lieshout 2001).

Factors that affect the bioavailability and bioconversion of carotenoids

The bioavailability and bioconversion of carotenoids are affected by various factors including the type of carotenoid, the food matrix, the amount of carotenoid consumed, effectors of absorption and conversion, the nutritional status of the subject, and genetic factors (Castenmiller and West 1998).

Previous human studies showed that the all-*trans* form of β-carotene was absorbed more easily than the 9-*cis* form (Ben-Amotz 1996). It was assumed that β-carotene had twice the vitamin A activity as other provitamin A carotenoids, including the *cis* isomers of β-carotene (FAO/WHO 1998).

The amount of carotenoids consumed in a meal affects both bioavailability and bioconversion. A study showed when a dose of β-carotene supplement was divided into three times and taken daily, serum β-carotene concentration was increased three times more than same dose taken once daily (Prince and Frisoli 1993).

Food matrix lowers the bioavailability of β-carotene to different extents, compared with the bioavailability of β-carotene dissolved in oil. The serum retinol response to ingestion of fruit β-carotene was found to be four times the serum retinol response to ingestion of vegetable β-carotene (De Pee 1998). Proteins in chloroplasts as well as cell structures entrap the carotenoids and decrease the bioavailability of carotenoids from

vegetables with dark-green leaves (Castenmiller and West 1998). Cooking releases carotenoids from protein and cell structures, which largely increases carotenoid bioavailability (Erdman 1988). However, the effect of food matrix is limited to the lumen of the small intestine. Therefore, food matrix effects on bioconversion are mostly attributed to the impact on the bioavailability of carotenoids (Castenmiller and West 1998).

The amount of dietary fat has a major impact on the absorption of carotenoids. The absorption of carotenoids in vegetables will be markedly improved when consumed with a small quantity of fat (Roels 1958). At least 5 g fat per meal was considered to be necessary for optimal absorption (Prince 1993, Jialal 1991, Jayarajan 1980). However, when the amount of consumed dietary fat is increased, the bioavailability of different types of carotenoids will not be affected equally (Yonekura and Nagao 2007). As β-carotene and α-carotene are highly lipophilic carotenes, while lutein is relatively polar. Under limited oil conditions, the micellization of highly lipophilic carotenes will be inhibited compared with lutein, which transfers more freely into the lipid phases in the digesta to form micelle fractions. For example, in an *in vitro* digestion study, when spinach puree was mixed with 2.1-3.5% fat, the micellization rate of lutein was about twice of the rate of α-carotene and β-carotene (Garrett et al 1999, Chitchumroonchokchai et al 2004, Garrett et al 2000). When the fat content was increased to about 10% in spinach puree for a simulated digestion, the micellization of lutein and β-carotene was almost equal (Ferruzzi et al 2001).

Dietary fiber interacts with bile acids, reduces the reabsorption of bile acids and fats, and affects the absorption of fat-soluble substances. A study showed that serum β-carotene content was decreased by 42% when pectin was added into the diet (Rock et al 1992).

Other factors consumed with carotenoids also affect bioavailability and bioconversion. Protein increases micelle formation and carotenoid absorption by stabilizing the lipids and facilitating the formation of a fat emulsion (Castenmiller and West 1998). Interactions with drugs and some ingredients from food in the gastrointestinal tract, such as sulfides and acids, may decrease the bioavailability of carotenoids (Peiser et al 1979, Wedzicha et al 1983).

The nutrient status of the host also affects the bioavailability and bioconversion of carotenoids. High supplementation with vitamin A decreased the absorption of both β-carotene and canthaxanthin (Sklan et al 1989). In a study with vitamin A-replete subjects, after ingestion of a single 40 mg β-carotene dose, only 22% was absorbed and 18.5 µg dietary β-carotene was equivalent to 1µg of retinol (Novotny et al 1995).

Genetic factors may affect β-carotene levels according to various studies. After adjusting for influencing factors, data showed that, among hemodialysis patients, African Americans had higher plasma β-carotene concentrations than white patients (Rock et al 1997). Another study showed Nigerian women had 1.2-13 times greater β-carotene levels and 2.5 times greater total carotenoid levels than those of the US population (Adams-Campbell et al 1992). The efficiency of the enzyme β-carotene 15,15' monoxygenase (BCMO1), which is responsible for β-carotene conversion into retinal, is also affected by its genetic polymorphism. Two common nonsynonymous single nucleotide polymorphisms were identified in the BCMO1 coding region among subjects. Carriers of both variant alleles were found to have a reduced ability to convert β-carotene to vitamin A (Leung 2009**)**.

Retinol activity equivalence

The US Institute of Medicine established retinol activity equivalence (RAE) values in 2001 to express vitamin A requirements. On average, consumption of 1 µg preformed

vitamin A equivalents yields 1 µg of bioavailable retinol. A 2 µg supplemental β-carotene dose yields 1 µg retinol, while β-carotene from fruits and vegetables has much lower bioavailability, which requires 12 µg to yield 1 µg retinol. Thus the bioavailability of β-carotene in fruits and vegetables is considered to be on average 6 times lower than that of a β-carotene supplement. Based on the molecular structure, 24 µg α-carotene or 24 µg β-cryptoxanthin is needed for 1 µg bioavailable retinol.

Vitamin A deficiency

Fat-soluble vitamin A (retinol) and its derivatives (retinal and retinoic acid) are essential for cell differentiation, proliferation and signaling, and they play a critical role in vision, reproduction, and immune functions (Dowling et al 1958, Parker 1985, Sommer 2008).

Although vitamin A deficiency is rarely seen in developed countries, it is a common public health problem in developing countries due to diets poor in vitamin A. Preschool children and pregnant women are most vulnerable to vitamin A deficiency (Maziya-Dixon et al 2006). It is estimated that vitamin A deficiency affects 75 to 252 million preschool children each year (Maziya-Dixon 2006). Night-blindness is the first symptom of vitamin A deficiency in humans and other animals, which means patients need higher levels of illumination to see (Dowling and Wald 1960). If vitamin A deficiency gradually grows worse, another ocular problem called xerophthalmia or “dry eye” will develop and ultimately lead to blindness. By dietary intervention, this eye problem had been eliminated from developed countries by the early 1940s (Sommer 1995). However, worldwide, about 4.4 million preschool children are still having vision problems caused by vitamin A deficiency. Every

year, about 250,000 to 500,000 children become blind and two thirds die within months after that (West 2002). Moreover, vitamin A deficiency may also result in decreased immunity, and increased morbidity and mortality (Dowling et al 1958, Parker 1985, Sommer 2008). A study in Nepal showed dosing women of childbearing age with either vitamin A or β-carotene resulted in lower mortality related to pregnancy (West et al 1999). However, impoverished populations heavily rely on plant sources of provitamin A carotenoids to meet their vitamin A requirement (West et al 2003).

Currently two biochemical indicators are used to define vitamin A status: serum retinol and serum retinol-binding protein (de Pee and Dary 2002). According to the World Health Organization, vitamin A deficiency is defined as serum retinol concentration lower than 0.70 µmol/L, based on data collected from a large variety of populations (WHO/United Nations 1994). Compared with retinol, which needs to be detected in the laboratory with precise analytical instruments such as HPLC, serum RBP is easier and much cheaper to be assessed even in the field, and correlates well in a 1:1 molar ratio with retinol (de Pee and Dary 2002). However, not all RBP found in serum is bound with retinol, and their relationship is affected by many factors in different populations. Current proposed cut-offs for serum RBP vary considerably among different populations. Therefore, there is no well-founded or reproduced cut-off for serum RBP concentration to reflect vitamin A deficiency in terms of a serum retinol concentration lower than 0.7 µmol/L. However, since the concentration ratio between serum RBP and retinol is stable, the former can serve to determine whether vitamin A deficiency is a public health problem, when the relationship between the two has been defined in a specific population (de Pee and Dary 2002).

Vitamin A biofortification in cassava

The strategy of vitamin A biofortification

To combat vitamin A malnutrition in developing countries, current efforts have focused on providing vitamin A supplements for children and pregnant women, processing fortification, and vitamin A-biofortification.

Biofortification is the process to develop staple crops containing high micronutrient contents using conventional plant breeding technology and/or modern biotechnology (Nestel 2006). Through biofortification, crops will be loaded with high levels of minerals and vitamins in their seeds and roots, which will provide multiple benefits. Staple crops are the predominant food source for many low-income families in developing countries. The strategy of biofortification of regularly consumed staples crops targets every family member in micronutrient-deficient populations (Nestel 2006). Biofortification is a cost-effective and sustainable process. Once the biofortified crops are developed, they will be able to be grown and to produce more seeds and roots. The seeds or roots can be shared internationally to improve the nutritional status of people all over the world, even if the formal investment in biofortification research ends. Current biofortification research efforts include developing genotypes of cassava with higher provitamin A carotenoid contents in the starchy roots. Subsequently, the germplasm of those varieties will be shared within the developing world (HarvestPlus 2006).

Biofortification requires multidisciplinary collaboration among scientists to communicate and cooperatewith innovative strategies to produce biofortified seeds and roots. The lead organizations in the HarvestPlus biofortification program include the Consultative

Group in International Agricultural Research (CGIAR) and the National Agricultural Research and Extension Services (NARES), which take local responsibility for plant breeding, to develop varieties with both nutritional and agronomic value, for crops such as bean, cassava, maize, rice, sweet potato and wheat. Food science and human nutrition researchers are also involved in biofortification research. They study the retention of nutrients after processing and cooking, the bioavailability of micronutrients, and the bioefficacy of the biofortified crops in human subjects. Biotechnology scientists are involved in biosynthetic genetics and study pathways that impact nutrient absorption (Nestel et al 2006).

Although micronutrients account for a small proportion of the mass of the seeds, whether such a small proportion will change the appearance, flavor, texture or processing methods needs to be investigated. It is desirable to biofortify crops to achieve high micronutrient content. However, it is also necessary to ensure that the fortified products will be adopted by farmers and consumers. Increased β-carotene content is always associated with yellow color in crops such as sweet potato and cassava. This might reduce the consumers' preference when choosing between traditional white products and biofortified yellow products. But with education, the consumers can learn that yellow color often indicates higher β-carotene content and be persuaded to choose yellow products with more health benefit (Bouis 2003).

Cassava production and consumption

Cassava (*Manihot esculanta*) is a woody plant of the *Euphorbiaceae* (spurge) family. Typically, cassava has white flesh, and depending on the cyanogenic content, it can be either sweet or bitter. Cassava serves as a major energy source for about 500 million people in

tropical and sub tropical regions, because it grows well under marginal conditions of soil and climate, survives diseases and pest attack and yields more energy per hectare compared with other crops (Montagnac 2009).

About 70 percent of the cassava production in the world comes from five countries including Nigeria, Thailand, Brazil, the Congo Democratic Republic and Indonesia. Based upon an area expansion at a rate of 1.8 percent a year, total world cassava production increased 2.2 percent per year since 1984, and reached 164 milliontons in 1997. However, the yield increased very slightly at only 0.4 percent a year (Food and Agriculture Organization, 2000).

The provitamin A carotenoid in biofortified cassava is primarily β-carotene. In white cassava, there may be trace amounts of β-carotene, which may be present in concentrations as low as 1 mg/g fresh weigh or 3 mg/g dry weigh (Iglesias et al 1997, Chavez et al 2005).

There are several traditional cassava processing techniques according to different needs, including roasting, boiling, frying, sun-drying, grating, pounding, soaking and etc. Sweet cassava roots are often boiled and eaten either hot or cold (Lancaster 1982).

Cooking and processing methods can affect the retention of β-carotene in cassava. Gari, a popular West African cassava dish processed through fermentation and gelatinization, has a β-carotene retention of about 32%. A 90% decrease of β-carotene content was reported after 20 minutes roast fermenting at 195°C. However, a lower roasting temperature at 165°C and a shorter time for 10 minutes resulted in 63% retention (Thakkar et al 2009). Other methods, including boiling, oven-drying and sun-drying all reduced β-carotene retention at different levels, ranging from 20 to 90 percent. As for boiled cassava, the retention is about 90% according to an *in vitro* digestion study (Thakkar et al 2009).

Cassava cyanogen removal

Cassava contains cyanogenic glucosides in the form of linamarin and lotaustralin, which give cassava a bitter flavor (Zvauya et al 2002). Linamarin and lotaustralin are broken down during processing into cyanohydrins and methylethyl ketone, respectively, and later volatilize as hydrogen cyanide (Sopade et al 2000). Inadequately processed cassava will cause cyanide poisoning and is linked to diseases like *konzo* (Casadei 1990).

There are several treatments to reduce the cyanogen content in cassava. Sun-drying of peeled cassava roots is the easiest way, so that it is popular in many tropical areas. The sun-dried roots are convenient for storage and transportation, and available for further processing before consumption (Essers 1996). However, high residual cyanogen levels in sun-dried cassava were detected, indicating ineffective cyanogen removal (Pieris et al 1974). Effective cyanogen removal requires two treatments. The first step is to increase linamarin-linamarase contact and to enhance enzyme activity, by crushing or grating the cassava into flour. The second treatment is to degrade the cyanohydrins and volatilize them in the form of HCN, by heating or drying (Essers et al 1996). A wetting/spreading method was considered as feasible and effective for cyanogen removal. In this method, cassava dough was prepared by mixing cassava flour with water. The dough was left for 5 hours at 30°C, which resulted in an average of 16.7% retention of hydrogen cyanide. A large flour mass might cause the accumulation of HCN and thus affect the pH and reduce the breakdown efficiency of linamarin catalysed by linamarase. However, this problem can be overcome by spreading the cassava dough on a tray into a layer about 0.5 cm thick (Cumbana et al 2007).

Provitamin A biofortification of cassava

Current research focuses on developing genotypes of cassava with higher provitamin A carotenoid contents in the roots. And, subsequently, the germplasm of those varieties will be shared within the developing world. Hybridization of promising cassava varieties usually yields a large number of seeds, and high quality hybrids are selected after large-scale seeding by color and agronomic performance. Carotenoids from cassava oxidize easily due to exposure to light, air, and physical damage, which means the postharvest losses of provitamin A should not be ignored (Thakkar et al 2009).

The bioavailability of β-carotene from different sources varies considerably (Reboul et al 2006). Therefore it is essential to measure the vitamin A equivalence of the β-carotene in β-carotene-biofortified cassava before breeding efforts continue. Several methods have been reported to be involved in studying the bioavailability of provitamin A carotenoids in food products such as cassava, including a stable-isotope reference method, *in vitro* digestion, and an animal model. In vitro digestion using Caco-2 cells showed that, in boiled cassava and gari, the bioaccessibility of all-trans and cis isomers of β-carotene was 25-30%, while it was only 12-15% in fufu (fermented and cooked cassava paste) (Thakkar et al 2009). An appropriate animal model is an effective and low-cost alternative to study the bioavailability of provitamin A carotenoids by directly measuring liver vitamin A (Howe 2009), which is considered the best indicator of vitamin A status (Goodman 1984). It was reported in a gerbil model, that biofortified cassava adequately maintained vitamin A status, as effectively as β-carotene supplementation (Howe et al 2009). The vitamin A equivalence of spirulina β-carotene in human subjects was studied by a stable-isotope reference method in which spirulina was grown in $^{2}H_2O$ culture solution to obtain the greatest possible [$^{2}H_{10}$] trans β-carotene enrichment (Wang et al 2008). However, it is not feasible to use this stable tracer

method in cassava roots because they are grown underground. In addition, intrinsic labeling of crops with stable isotope tracers is expensive, time consuming, labor intensive, and technically demanding. Therefore we introduced high sensitivity HPLC with coulometric array electrochemical detection (ECD) to detect the vitamin A concentrations in processed lipoprotein fractions from postprandial triacylglycerol-rich lipoprotein samples.

Electrochemical detection (ECD) is based on the oxidation and reduction of the compounds in the samples. It is able to detect compounds at the femtogram level. By using the multichannel electrochemical detector, various micronutrients elute separately under different potentials after being oxidized or reduced selectively. Interfering compounds are removed prior to the detection of the component of interest through selective oxidation (Ferruzzi et al 1998).

The objective of this study was to apply high-sensitivity coulometric array electrochemical detection to quantify the vitamin A equivalence of β-carotene-biofortified cassava in women based on the ingestion of a single realistic serving of cassava porridge.

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CHAPTER 2. VITAMIN A EQUIVALENCE OF THE β–CAROTENE IN BIOFORTIFIED CASSAVA IN WOMEN

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Abstract

Cassava is an important food energy source in tropical and subtropical regions. There have been successful efforts to increase the β-carotene content in cassava through biofortification. However, the bioefficacy of the β-carotene in the biofortified cassava in humans has not been studied. In the present study, we investigated the bioefficacy of the β-carotene in biofortified cassava in 9 healthy women ages 18-39 y. After eating conventional foods with low β-carotene and vitamin A contents for three days, the subjects were asked to ingest one of the three cassava porridges in random order. All of the porridge servings were about 200 g and contained 40 g cassava flour. The three porridges included biofortified cassava porridge (1097.5 µg β-carotene), white cassava porridge with vitamin A reference dose (285.6 µg retinol) and white cassava porridge with β-carotene reference dose (537.6 µg β-carotene). Blood samples were collected prior to ingestion of the test porridge and at 2, 3.5, 5, 6.5, 9 hours after ingestion. Plasma triacylglycerol rich lipoproteins (chylomicrons and large VLDL) were isolated and the retinyl palmitate contents were analyzed by HPLC-ECD. The mean amounts of retinyl palmitate appearing in the triacylglycerol-rich lipoprotein

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fraction in the entire plasma pool after ingestion of the biofortified cassava porridge and the white cassava porridge with the β-carotene reference dose were 1587.53 ± 285.27 and 914.35 ± 128.50 nmol. The vitamin A equivalence values of the β-carotene in these two porridges were 2.80 ± 1.77 to 1 and 2.11 ± 0.81 to 1 (by weight), respectively. These vitamin A equivalence values were not significantly different by t test. In our study population, the bioefficacy of the β-carotene in biofortified cassava was as good as that of a β-carotene supplement.

Key words: β-carotene, bioefficacy, biofortification, cassava, vitamin A equivalence,

Introduction

Vitamin A deficiency is a common public health problem in developing countries due to diets deficient in vitamin A. Preschool children and pregnant women are most vulnerable to vitamin A deficiency (World Health Organization 1995). It is estimated that vitamin A deficiency affects 75 to 252 million preschool children each year (Maziya-Dixon et al 2006). Vitamin A deficiency may result in blindness, night blindness, decreased immunity, and increased morbidity and mortality (Sommer et al 2008). To combat vitamin A malnutrition in developing countries, efforts have focused upon providing vitamin A supplements for children and pregnant women and vitamin A fortification. There is strong evidence that vitamin A supplementation programs have reduced mortality among infants and pregnant women (West et al 2003). However, impoverished populations heavily rely on plant sources of provitamin A carotenoids to meet their vitamin A requirements (West et al 2003). Biofortification is the process to develop staple crops containing high micronutrient contents using conventional plant breeding technology and/or modern biotechnology (Nestel et al

2006). Through biofortification, crops will be loaded with high levels of minerals and vitamins in their seeds and roots, which will provide multiple benefits. Staple crops are the predominant food source for many low-income families in developing countries. The strategy of biofortification of regularly consumed staples crops targets every family member in micronutrient-deficient populations (Nestel et al 2006). Biofortification is a cost-effective and sustainable process. Once the biofortified crops are developed, they will be able to be grown and to produce more seeds and roots. The seeds or roots can be shared internationally to improve the nutritional status of people all over the world, even if the formal investment in biofortification research ends. Current biofortification research efforts include developing genotypes of cassava with higher provitamin A carotenoid contents in the starchy roots. Subsequently, the germplasm of those varieties will be shared within the developing world (HarvestPlus 2006).

Cassava (*Manihot esculanta*) serves as a major energy source for about 500 million people in tropical and subtropical regions, because it grows well under marginal conditions of soil and climate, survives diseases and pest attack and yields more energy per hectare compared with other crops (Montagnac et al 2009). The provitamin A carotenoids found in cassava are primarily β-carotene. However, in white cassava, there may be trace amounts of β-carotene, which may be present in concentrations as low as 1 mg/g fresh weight or 3 mg/g dry weight (Iglesias et al 1997, Chavez et al 2005).

The bioavailability of β-carotene from different plant sources varies considerably and is unpredictable (Reboul et al 2006). Therefore it is essential to experimentally determine the vitamin A equivalence of the β-carotene in biofortified cassava. We introduced a highly sensitive HPLC detection technique, coulometric array electrochemical detection (ECD), to

quantify the minute vitamin A concentrations in the postprandial plasma triacylglycerol-rich lipoprotein fractions after subjects ingested a single realistic test meal (White et al, 2008). The objective of the current study is to quantify the vitamin A equivalence of the β-carotene in biofortified cassava based on the ingestion of a single realistic serving of cassava porridge.

Materials and methods

Subjects

Nine healthy, non-pregnant women ages 19 – 39 years were enrolled in the study. The subjects were screened by a standardized interview regarding diet, health history, lifestyle factors, and anthropometrics (height and weight). The exclusion criteria included smoking in the past 12 months, vegetarian diet, eating disorders, intestinal disorders, lactose intolerance, psychological aversion to phlebotomy, recent vitamin or mineral supplement use, use of medications which can affect lipid absorption or transportation, use of hormonal contraceptives over the past 12 months, history of anemia, low iron status or hemoglobin, excessive bleeding, chronic disease, lipid malabsorption, high plasma triacylglycerol or cholesterol concentrations, irregular menstrual cycle, body mass index (BMI $\geq$ 30), recent significant change in weight, and frequent consumption of alcoholic beverages (>1 drink/day). Informed consent was obtained from all subjects before the interview. After the interview, those subjects who appeared to qualify were invited to complete blood screening, which included plasma lipid, lipoprotein, and biochemistry profiles and a complete blood count. Study procedures were approved by The Human Subjects Research Review Committee of Iowa State University.

Test Meal

The β-carotene-biofortified cassava (yellow cassava) and the white cassava were grown under the same conditions at the International Center for Tropical Agriculture (CIAT), Cali, Colombia. Immediately after harvest, the roots were waxed and shipped to Iowa State University. The waxed roots were peeled, and the starchy endosperm was sliced. The slices were dried for 18 hours at 40°C in an oven (Model No. 1675, Sheldon Manufacturing, Inc., Cornelius, OR), milled (Grindomix GM200, Retsch GmbH Germany) into fine flour, and stored in the dark at -80°C until use.

A wetting method was used for to remove the cyanogens in the cassava flour (Cumbana et al 2007). Cassava flour (40 g) was mixed with water (50 g) to form wet dough, which was spread into a thin layer in an aluminum tray. The wet dough was heated at 30°C (Isotemp Oven, Fisher Scientific, Chicago, IL) for 5 hours. The dough was then held at 4°C for 12 hours overnight prior to use. The cyanogen content of the dough was determined by kit B2 (Bradbury et al 1999), which was purchased from Dr. J. Howard Bradbury, Australian National University, Canberra, Australia.

Cassava porridge was prepared according to a traditional African processing method (Lancaster 1982). The cassava dough was mixed with 30 g of cool water, and added to 90 g of boiling water in a Teflon-coated pan on the burner of a stove. After stirring for approximately 1 minute, the porridge was transferred to a serving bowl. Sugar (14 g) was added to each serving of test porridge. Prior to ingestion, 8 g of high oleic acid sunflower oil (Spectrum Organic Inc, Haines Celestial Group, Inc., Melville, NY) was added to the test porridges. In the case of the white cassava porridge with β-carotene reference dose, the sunflower oil contained 537.6 µg β-carotene. In the case of the white cassava porridge with

vitamin A reference dose, the sunflower oil contained 285.6 µg retinol in the form of retinyl palmitate. The cassava porridges were then immediately ingested by the subjects.

The commercial formulations used to prepare the vitamin A and β-carotene reference doses were donated by DSM Nutritional Products, Ltd (Basel, Switzerland). The starting concentrations of vitamin A and β-carotene were 1.7 MIU/g (510,000 µg RAE/g) in the form of retinyl palmitate and 30% (300,000 µg/g), respectively. These commercial formulations were diluted with the high oleic acid sunflower oil. The final concentration of and the β-carotene reference dose in sunflower oil was 67.2 µg/g. The final concentration of the vitamin A reference dose in sunflower oil was 35.7µg/g. The final concentrations were confirmed by HPLC with UV/VIS detection.

Experimental design

The subjects were assigned to one of the three test porridges in random order during each of the three one-week study periods. Each study period was separated by a washout period of at least one week.

During each of the three study periods, the subjects were instructed to follow the following protocol. Days 1-3: avoid provitamin A carotenoid- and vitamin A-rich foods (a list of foods to avoid was provided); Days 4-6: consume a standardized, weighed, low-vitamin A diet consisting of conventional foods at the Iowa State University Nutrition and Wellness Research Center (NWRC). Breakfast and dinner were consumed under supervision. A lunch and snack was carried out. The meals were low-carotenoid and low-vitamin A to reduce variability among subjects and to induce intestinal vitamin A depletion. Day 7: Subjects returned to the NWRC in the morning after an overnight fast. A 40 mL baseline blood sample was collected from a forearm. The subjects then ingested one of the three test

porridges followed by 240 mL of bottled water. Additional blood samples were collected at at 2, 3.5, 5, 6.5, and 9 hours after ingestion of the test porridge. A vitamin A free snack (5 Nabisco® Nilla Wafers) was provided after the 3.5 hour blood draw. After the 5 hour blood draw, a small low-fat lunch was provided, which consisted of 48 g Rotella® (Omaha, NE) Italian Sandwich Bread (1.62 g fat) and 96 g Oscar Mayer® (Rye Brook, NY) Natural Turkey Breast (1g fat). The subjects were not permitted to drink water again until after the 3.5 h blood draw.

Subjects remained on the premises of the Nutrition and Wellness Research Center. Dinner was provided after the last blood draw. Subjects could then resume their normal diet and activities until the next study period.

Sample collection and treatment

Blood samples were collected via butterfly needle blood collection sets (BD Vacutainer Brand, REF# 367283, Franklin Lakes, NJ) into 10 mL plastic vacutainer tubes sprayed with anticoagulant K_2EDTA (BD Vacutainer Brand, Catalog # 366643, Franklin Lakes, NJ). Blood samples were held on ice and then centrifuged at 700 × g for 20 minutes to separate plasma.

Plasma (4 mL) was transferred to a 14 × 95 mm Ultra-Clear centrifuge tube (Catalog# 344060, Beckman Instruments, Inc, Spinco Division, Palo Alto, CA). Plasma chylomicron and large VLDL (VLDLA) fractions were isolated by cumulative rate ultracentrifugation (Redgrave et al 1979, Lindgren et al 1975). For each milliliter of plasma, 0.14 g potassium bromide (Fisher Scientific, Chicago, IL, catalog # P205-500) was added to adjust the density to 1.10 g/mL. To prevent carotenoid and retinoid degradation, all procedures were performed under yellow light. Three density solutions were prepared for chylomicron and VLDLA

isolation (Redgrave et al 1979, Lindgren et al ,1975), including 1.006 g/mL sodium chloride (Fisher Scientific, Chicago, IL, Catalog # S271-500) stock solution at pH 7. In addition, 1.020 g/mL and 1.065 g/mL potassium bromide solutions were prepared from the 1.006 g/L stock solution. The densities were confirmed using a digital density meter (DMA 48, Anton-Paar USA, DMA-48, Ashland, VA). After the density-adjusted plasma was transferred to an Ultra-Clear centrifuge tube, the density solutions described above were added into the tube carefully in the following order. First, 3 mL of 1.065 g/mL density solution was added, and then overlaid with 3 mL of 1.020 g/mL density solution. Finally, 2 mL of 1.006 g/mL density solution was added as the upper layer. The fill lines were marked after adding the second and third solutions. The samples were then centrifuged in a swinging bucket rotor (SW 40Ti, Beckman Instruments, Inc) in an ultracentrifuge (Model L8-70M, Beckman Instruments, Inc). The chylomicron fraction was isolated as the top fraction in the 1.006 g/mL solution, as delineated by the two fill lines, after centrifugation at 28,300 rpm (101136 g) for 43 minutes at 20°C. This fraction was removed and stored at -70°C until analysis. The tube was refilled with another 2 mL of 1.006 g/mL density solution. The VLDLA fraction was then isolated by centrifugation at 40,000 rpm (142948 g) for 67 minutes at 20°C. This fraction was then removed from the top of the centrifuge tube and stored at -70°C until analyzed.

Analysis of retinyl palmitate and β-carotene in the chylomicron and VLDLA fractions

The quantities of the absorbed β-carotene and its major vitamin A metabolite, retinyl palmitate, were analyzed by HPLC with coulometric array electrochemical detection (HPLC-ECD). The volumes of the chylomicron and the VLDLA fractions at each time point were each approximately 2 mL. An equal volume of methanol was added to the entire fraction at each time point for deproteination, followed by three extractions with 4 mL of hexane (0.01%

BHT) to extract carotenoids and retinoids. The collected hexane layers were combined and dried using a speed vacuum evaporator (Model SPD 131 DDA, Thermo Electron Corporation, Milford, MA) with a universal vacuum system (UVS 800 DDA, Thermo Electron Corporation, Milford, MA). The sample was reconstituted in 30 µL methanol and 30 µL MTBE and 25 µL was analyzed by HPLC-ECD. The HPLC-ECD system included a C_{30} analytical Carotenoid Column (4.6 × 250 mm, Waters, Milford, MA), two solvent delivery systems (Model 582, ESA, Chelmsford, MA), an autosampler (Model 542, ESA) , a Coularray® Electrochemical detector with eight channels, a thermal organizer and an ESA Model 5600 Coularray® Electrochemical Detector. Data collection and integration were performed by ESA Coularray® software (Coularry Win 2.0). The mobile phases consisted of methanol (EMD Chemicals Inc. Gibbstown, NJ, catalog #: MX0488-1), methyl-tert-butyl ether (MTBE, Sigma, St. Louis, MO, catalog #: 650560), and aqueous ammonium acetate buffer (1M, pH 4.6) (Sigma, St. Louis MO, catalog # 17836). The gradient consisted of mobile phase A with methanol:MTBE:buffer (95:3:2 by volume) and mobile phase B with methanol:MTBE:buffer (25:73:2 by volume). The gradient started with a 1-minute hold at 100% mobile phase A. Over the next 40 minutes, the gradient linearly changed to 25% mobile phase A and 75% mobile phase B with a stable total flow rate of 1 mL/min. The autosampler held samples at 4 °C and injected the samples by the microliter pick up method with mobile phase A as the transport solvent. The potentials in the eight channels in the detector were set at 100 mV, 300 mV, 400 mV, 450 mV, 550 mV, 600 mV, 750 mV and 800 mV, respectively. The dominant channel for analysis of β-carotene was 400mV and the dominant channel for analysis of retinyl palmitate was 750 mV. The thermal organizer was

used to heat the analytical column to 33°C. Retinyl palmitate (Sigma Chemical) and β-carotene external standard curves were used for quantification.

HPLC-UV/VIS analysis of the carotenoids in the test porridges

To analyze the carotenoid contents in the cassava flours, wet doughs and test porridges, a 1.0 g sample was weighed into a 50 mL screw capped glass tube. Then 6 mL methanol containing 0.01% butylated hydroxytoluene (BHT) was added, as well as an internal standard, β-apo-8'-carotenal (Fluka Chemical Inc). After vortexing for 90 seconds, samples were centrifuged for 5 min at 700 × g. The upper organic phase was collected into a 25 mL volumetric flask. The sample was extracted again with 6 mL methanol (0.01% BHT) and 6 mL tetrahydrofuran (THF, VWR, Boston, MA containing 0.01% BHT). After vortexing for 90 seconds and centrifuging for 5 min at 700 × g, the upper organic phase was collected and combined into the same 25 mL volumetric flask. The sample was then extracted a third time with 6 mL of THF. Additional THF (0.01% BHT) was added to bring the volume to 25 ml in the volumetric flask. A 1 mL aliquot of the extract was dried in a speed vacuum evaporator (Model SPD 131 DDA, Thermo Electron Corporation, Milford, MA) with a universal vacuum system (UVS 800 DDA, Thermo Electron Corporation, Milford, MA), and reconstituted with 300 µL methanol and 100 µL MTBE. A 100 µL aliquot was injected into the HPLC-UV/VIS system. All samples were extracted and analyzed in duplicate.

Carotenoids were separated with a 5-µm analytical Carotenoid Column (4.6 × 250 mm, Waters Corporation, Milford, MA) and detected by a 2966 Photodiode Array Detector (Waters Corporation). The temperature of the 717 Plus Autosampler was set as 5°C. Two mobile phases were pumped separately by two 515 HPLC pumps. Mobile phase A consisted

of methanol (HPLC grade, Fisher Scientific, Chicago, IL) containing 0.1% ammonium acetate (Fisher Scientific). Mobile phase B contained 100% MTBE (HPLC grade, Fisher Scientific). Mobile phases were filtered with a 0.2 µm, 47 mm nylon membrane filter (Whatman International Ltd, Maidstone, England) prior to use. The gradient started with 100% mobile phase A at a flow rate of 1.0 mL/min, and linearly increased to 100% MTBE over 70 minutes. The β-carotene peak was integrated at 453 nm, a commercial β-carotene standard (Carotenature, Lupsingen, Switzerland) was used to generate the internal standard curve for calibration.

The moisture contents of the porridges were analyzed by Association of Official Analytical Chemists (AOAC) Method 925.10 for solids (total) and loss on drying (moisture) in flour (AOAC, 1925).

HPLC-UV/VIS analysis of the β-carotene and vitamin A reference doses

The concentrations of β-carotene and vitamin A in the corresponding reference doses were confirmed by HPLC-UV/VIS analysis. A 0.1 g aliquot of the freshly prepared reference dose was weighed into a 50 mL screw capped glass test tube and votexed with 6 mL methanol (0.01% BHT) and 6 mL tetrahydrofuran (0.01% BHT). An 0.5 ml aliquot was then transferred into a 15 ml screw capped glass test tube. Then 1 mL of freshly prepared 40% potassium hydroxide in methanol with 0.1 M pyrogallol was added and the tube was vortexed for 2 min. The saponification step was followed by a washing step in which 2 mL HPLC grade water were added and vortexed for 30 s. The internal standard (150 ng) in methanol was added to the tube, as well as 4 mL hexane:methylene chloride (5:1 by volume). The tube was vortexed again before a 5 min centrifugation at 700 × g. The organic phase was collected and washed again with 1 mL water. The organic phase was collected again after

another 5 min centrifugation step. The β-carotene reference dose was analyzed using the same mobile phase gradient previously described for analysis of the test porridge with an internal standard β-apo-8'-carotenal. Retinol was eluted isocratically using 90% acetonitrile and 10% water as mobile phase with retinal added as an internal standard.

Data analysis

The postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate contents at each time point were calculated from the area counts of the retinyl palmitate peaks on the dominant channel (750 mV) after HPLC-ECD analysis. An external standard curve, as well as the area of each peak, were used to calculate the amount of retinyl palmitate in the chylomicron and the VLDLA fractions. The contents of retinyl palmitate in the chylomicron and VLDLA fractions were summed at each time point. The total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate content in the entire plasma pool was calculated by multiplying the summed retinyl palmitate content in the chylomicron and VLDLA fractions from one liter of plasma (nmol/L plasma) with the calculated plasma volume for each subject (0.0427L*kg body weight) (Boer, 1984; Tang et al., 2005).

The absorption curves were plotted with the baseline-adjusted total plasma TRL content of retinyl palmitate (nmol) at each time point. The area under the curve (AUC) vaules (nmol·h) were calculated by the trapezoidal method using the absorption curve with TRL content of retinyl palmitate (nmol) as y axis and the time (hour) as the x axis. The AUC was calculated using SAS 9.1.3 (Cary, NC).

With the AUC results, we applied the calculations described by Tang et al. (2005): Vitamin A (nmol) formed from the biofortified cassava =

$$\frac{\text{Retinyl palmitate AUC after ingestion of biofortified cassava}}{\text{Retinyl palmitate AUC after ingestion of white cassava with vitamin A reference dose}} \times \text{vitamin A reference dose (nmol)} \quad (1)$$

Vitamin A equivalence of provitamin A carotenoids in biofortified cassava to retinol (by wt) =

$$\frac{\text{β-Carotene equivalents in biofortified cassava porridge (nmol)} \times 536.8}{\text{Vitamin A formed from after ingestion of biofortified cassava (nmol)} \times 286.5} \quad (2)$$

where β-carotene equivalents = *trans* β-carotene + (9-*cis* + 13-*cis* β-carotenes)/2 (536.8 and 286.5 are the molecular weights of β-carotene and retinol, respectively.)

Equations (1) and (2) may be condensed into the following equation:

Vitamin A equivalence value of biofortified cassava =

$$\frac{\text{Retinyl palmitate AUC after ingestion of white cassava with vitamin A reference dose}}{\text{Retinyl palmitate AUC after ingestion of biofortified cassava}} \times \frac{\text{β-Carotene equivalents in biofortified cassava porridge (ng)}}{\text{Vitamin A in white cassava porridge with vitamin A reference (ng)}} \quad (3)$$

However, we need to correct for the trace amount of β-carotene in the white cassava flour in the white cassava porridge with the vitamin A reference dose. We assume that the vitamin A equivalence of the β-carotene in white cassava is the same as the vitamin A equivalence of the β-carotene in the biofortified cassava. It follows that the postprandial plasma vitamin A from the white cassava itself after ingestion of the white cassava porridge with vitamin A reference dose equals the β-carotene equivalents in the white cassava/the vitamin A equivalence of the biofortified cassava. The vitamin A in the white cassava porridge with vitamin A reference dose equals the vitamin A reference dose + (β-carotene

equivalents of white cassava/vitamin A equivalence of biofortified cassava). As a result, the corrected equation to calculate the vitamin A equivalence of the biofortified cassava is:

Vitamin A equivalence of biofortified cassava =

$$\frac{\text{Retinyl palmitate AUC after ingestion of white cassava with vitamin A reference dose}}{\text{Retinyl palmitate AUC after ingestion of biofortified cassava}} \times \frac{\text{β-Carotene equivalents in biofortified cassava porridge (ng)}}{\text{Vitamin A reference dose} + \frac{\text{β-carotene equivalents of white cassava}}{\text{vitamin A equivalence of biofortified cassava (ng)}}} \quad (4)$$

The solution of this equation is:

Vitamin A equivalence of biofortified cassava =

$$\frac{\left(\frac{\text{Retinyl palmitate AUC after ingestion of white cassava with vitamin A reference dose}}{\text{Retinyl palmitate AUC after ingestion of biofortified cassava}} \times \text{β-Carotene equivalents in biofortified cassava porridge (ng)} - \text{β-Carotene equivalents of white cassava} \right)}{\text{Vitamin A reference dose (ng)}} \quad (5)$$

Meanwhile, we also need to take into consideration the AUC formed by the β-carotene in the white cassava itself when calculating the vitamin A equivalence of the β-carotene in the white cassava porridge with β-carotene reference. As mentioned above, the vitamin A equivalence of β-carotene in white cassava is assumed to be the same as that of biofortified cassava. Therefore, from equation (4), the area under curve (AUC) formed from β-carotene in white cassava is:

Retinyl palmitate AUC formed by carotenoids in white cassava porridge =

$$\frac{\text{Retinyl palmitate AUC after ingestion of white cassava with vitamin A reference dose}}{\text{Vitamin A equivalence of biofortified cassava}} \times \frac{\text{β-Carotene equivalents of carotenoids from white cassava porridge (ng)}}{\text{vitamin A reference dose} + \frac{\text{β-carotene equivalents of white cassava}}{\text{vitamin A equivalence of biofortified cassava (ng)}}} \quad (6)$$

Therefore, the actual AUC value formed from the β-carotene reference dose after ingesting white cassava with the β-carotene reference dose is:

Retinyl palmitate AUC after ingestion of white cassava porridge with β-carotene reference dose − retinyl palmitate AUC formed from the β-carotene in the white cassava porridge (7)

Therefore:

RAE of β-carotene in white cassava porridge with β-carotene reference dose=

$$\frac{\text{Retinyl palmitate AUC after ingestion of white cassava with vitamin A reference dose}}{\text{Retinyl palmitate AUC formed from β-carotene reference dose after ingestion of white cassava with β-carotene reference dose}} \times \frac{\text{β-Carotene reference dose (ng)}}{\text{Vitamin A reference dose} + \frac{\text{β-Carotene equivalents in white cassava}}{\text{vitamin A equivalence of biofortified cassava (ng)}}} \quad (8)$$

Results

Reference dose concentration analysis and calculation

The retinyl palmitate and β-carotene reference doses were prepared by diluting the commercial formulations with sunflower oil. The final concentrations were confirmed by HPLC, and compared with the expected concentrations based upon the manufacturer's data

and the extent of dilution (Table 1). The retinyl palmitate and β-carotene concentrations in the reference dose were consistent across three independently prepared samples, and agreed with the expected concentrations. Therefore, the mean concentration of each reference dose was used in the calculations of the vitamin A equivalence values for the biofortified maize and for the β-carotene reference dose.

Efficiency of cyanogen removal in cassava

The wetting/spreading method effectively reduced the cyanogen content in the cassava flour as shown in Table 4. To test the efficiency of this method, we started with four kinds of cassava flours with different levels of cyanogen. The cyanogen removal rates were approximately 93% from cassava dough to porridge. Moreover, we showed that, after the cassava dough was spread on a tray and heated for 5 hours at 30 °C, the cyanogen contents in the four samples were lowered by at least 87%. The cyanide contents in test porridges were analyzed as well. As shown in Table 5, average cyanide content in yellow cassava and white cassava porridges were 0.99 ± 0.14 mg HCN/kg porridge, and 3.30 ± 1.19 mg HCN/kg porridge, respectively. Therefore cyanide contents in the test porridges were low enough to be considered as safe.

Carotenoid and moisture contents in cassava

There were several isomers of β-carotene in the cassava flours, including all-*trans*-β-carotene, 13-*cis*-β-carotene and 9-*cis*-β-carotene (Figure 1). In the test porridge prepared with β-carotene-biofortified cassava flour, the amounts per 200 g serving of *trans*-, 13-*cis*, and 9-*cis* β-carotenes were 970.71 ± 70.12 μg, 118.65 ± 0.69 μg, and 134.93 ± 0.49 μg, respectively. In the porridge prepared with white cassava flour, there were trace amounts of β-carotenes.

The amounts per 200 g serving of *trans*-, 13-*cis*, and 9-*cis* β-carotenes were 34.50 ± 1.00 μg, 17.57 ± 0.26 μg and 17.28 ± 0.45 μg, respectively. The moisture contents of the white cassava porridge and the biofortified cassava porridges were not significantly different and were approximately 81% (Table 2). Trans-, 13-cis, and 9-cis β-carotene contents were not statistically significantly different in both yellow and white cassava porridges. The macronutrient compositions of the flours were determined by Covance® (Madison, WI), and results are shown in Table 3.

Postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate responses

The postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate responses were detected by HPLC-ECD. The chylomicron and large VLDL (VLDLA) fractions were analyzed. Figure 2 shows a representative chromatogram of an extract of a postprandial chylomicron sample. The predominant response for β-carotene was at 400 mV applied potential on channel 3, and the retention time was about 31 minutes. The predominant response for retinyl palmitate was at 750 mV applied potential on channel 7, and the retention time was approximately 24 minutes.

Quality control (QC) materials were aliquots from a combined chylomicron and VLDLA pool isolated from extra plasmas collected during the study. We first determined the within-assay mean and precision of the HPLC-ECD analysis of the β-carotene and retinyl palmitate in the quality control material (Table 6). The within-assay coefficient of variation (CV) for retinyl palmitate and β-carotene weight in the QC material were less than 6%, which indicated excellent analytical precision. We also determined the inter-assay precision of the QC material (Table 7). During each run, one quality control sample was extracted and analyzed by HPLC-ECD together with that day's sample set. The inter-assay coefficient of

variation of the retinyl palmitate and β-carotene weight in the quality control samples were also less than 6%. There was good agreement between the inter-assay QC retinyl palmitate values and the intra-assay mean value that was previously determined for retinyl palmitate in the QC material.

The absorption curves were plotted using the baseline-adjusted TRL content of retinyl palmitate (nmol) in the entire plasma pool at each time point (Table 9-17, Figure 3-11). The time for peak absorptions varied somewhat among the individual subjects. Some subjects had a single absorption peak irrespective of the test porridge they ingested, and the plasma retinyl palmitate concentration returned to baseline gradually (Subject No. 3, No. 4). Some subjects had two absorption peaks (Subject No. 1, No. 2, No. 6, No. 9). However, for Subjects No. 5, No. 7 and No. 8, both patterns were observed in their absorption curves after ingestion one of the three test meals.

The area under the curve (AUC) values were used to calculate the vitamin A (nmol) formed from the β-carotene and the vitamin A equivalence of the β-carotene in the biofortified cassava porridge. The vitamin A equivalence of the β-carotene in the white cassava porridge with β-carotene reference dose was also calculated (Table 8). As shown in Table 8, the average vitamin A equivalence after ingestion of white cassava porridge with β-carotene reference dose was 2.11, which indicated that the average amount of supplemental β-carotene in oil to yield 1 µg of retinol was 2.11 µg. The average vitamin A equivalence value of the β-carotene in the biofortified (yellow) cassava porridge was 2.80, which indicated that, after ingesting each 2.80 µg β-carotene equivalents, 1 µg retinol will be formed in the body. A paired t test showed there was no significant difference in the mean vitamin A equivalence value of the β-carotene in the biofortified cassava porridge and the β-

carotene in the white cassava with β-carotene reference dose. Thus the bioefficacy of the β-carotene in the biofortified cassava porridge was as good as that of the β-carotene reference dose added to the white cassava porridge.

Discussion

The bioavailability of β-carotene-biofortified cassava has been studied both in *in vitro* digestion model and in an animal model (Thakkar et al. 2009; Howe et al. 2009) The *in vitro* digestion model used Caco2 cells to simulate small intestinal digestion of β-carotene, which provides a convenient and efficient approach to study the bioavailability of biofortified foods, including cassava. After simulated oral, gastric, and small intestinal digestion, it was discovered that the recovery of β-carotene from cooked cassava was over 70% (Thakkar et al., 2009). The percentage of β-carotene that was transferred into the micelle fractions during *in vitro* digestion was directly proportional to the β-carotene content but was unrelated to the genotype, isomeric structure, and total content of carotenoids.

However, the efficiency of bioconversion of absorbed β-carotene into vitamin A is not included in the *in vitro* digestion model, which is important when studying the bioefficacy of the provitamin A carotenoids in cassava (Reboul et al 2006). Moreover, the metabolism of β-carotene in humans is complex, and many factors that affect the bioavailability and bioconversion of β-carotene are not considered in the *in vitro* digestion model, including other foods consumed with the cassava, the nutritional status of the subject, and the amount of carotenoids consumed (Castenmiller and West 1998). By directly measuring liver vitamin A stores, which is considered the best indicator of vitamin A status (Goodman 1984), the gerbil model provided strong evidence that biofortified cassava was

able to adequately maintain vitamin A status as effectively as a β-carotene supplement (Howe et al 2009). However, to further and more accurately investigate the bioavailability of provitamin A carotenoids in β-carotene-biofortified cassava, a bioefficacy study in humans was needed. Because it is not feasible to biopsy the liver to determine liver vitamin A concentrations in human subjects, we detected newly absorbed vitamin A in the plasma triacylglycerol-rich lipoprotein fractions. Subjects were fed a low carotenoid and low-vitamin A-diet for 3 days preceding ingestion of the test porridge. The objective was to deplete the intestinal epithelium of vitamin A and induce a "pseudodeficiency" which would mimic true vitamin A deficiency. The changes of retinyl palmitate contents in the postprandial plasma were determined to study the efficiency with which β-carotene was converted into retinol in the body.

As established by the US Institute of Medicine (2001), on average, the retinol activity equivalence (RAE) value of supplemental β-carotene in oil is 2, which means that, on average, 2 µg of supplemental β-carotene is needed to yield 1 µg of retinol in the body. In our study, the average amount of supplemental β-carotene in oil needed to yield 1 µg of retinol was 2.11 µg, which was nearly identical to the number established by the US Institute of Medicine. This comparison validated our experimental approach. The average vitamin A equivalence value of the β-carotene in the β-carotene-biofortified (yellow) cassava porridge was determined to be 2.80. Thus after ingesting the β-carotene-biofortified cassava porridge, for each 2.80 µg of β-carotene equivalents ingested, 1 µg retinol will be formed in the body. There was no statistically significant difference in the vitamin A equivalence of the β-carotene in the yellow cassava porridge and the β-carotene in the white cassava porridge with β-carotene reference dose. We conclude that the bioefficacy of the β-carotene in the yellow

cassava porridge was as good as that of the β-carotene in the white cassava porridge with β-carotene reference dose. Thus our finding is consistent with that of the gerbil study described above (Howe et al., 2009).

Due to the trace amount of β-carotene content, which is as low as 1 mg/g fresh weigh or 3 mg/g dry weigh (Iglesias et al. 1997, Chavez et al. 2005), conventional white cassava has little value as a vitamin A source. Therefore tradition processing methods, including roasting, boiling, frying, sun-drying, grating, pounding, and soaking, do not consider preserving the β-carotene contents (Lancaster et al. 1982). Boiling, oven-drying and sun-drying each were reported to reduce the β-carotene contents to different extents, ranging from 20 to 90 percent (Thakkar et al 2009). In biofortified cassava, it is important to take into consideration the β-carotene retention, especially during the processing and cooking procedures. For example, a lower roasting temperature was reported to significantly increase β-carotene retention in cassava. The β-carotene retention increased from 10% to 63% after the roasting temperature was decreased from 195 °C to 165 °C and the roasting time was decreased from 20 minutes to 10 minutes (Thakkar et al 2009). In our study, it was shown that a low heating temperature (30°C) caused less than a 3% β-carotene loss although the heating time was as long as 5 hours (Table 1). When we boiled the cassava porridge for only 1 minute, the β-carotene loss was not significant (less than 2%) (Table 1). In general, it will be important to adjust processing and cooking methods to enhance β-carotene retention in cassava. Lower temperatures and shorter cooking times will enhance β-carotene retention.

Cassava contains cyanogenic glucosides in the form of linamarin and lotaustralin, which give cassava a bitter flavor (Zvauya et al 2002). Inadequately processed cassava will cause cyanide poisoning and is linked to diseases like *konzo* (Casadei et al 1990). Therefore

cyanogen removal is important prior to consumption. Two steps are necessary for effective cyanogen removal, including crushing or grating the cassava into flour, and heating or drying (Essers et al 1996). The wetting/spreading method was shown to be easy and effective (Cumbana et al 2007). Our results showed that the wetting/spreading method effectively removed about 87% of the cyanogen content (Table 3).

The newly absorbed vitamin A, in the form of retinyl esters, is mostly secreted in chylomicrons (Borel et al 1997). However, previous research showed vitamin A absorption is underestimated when assuming newly absorbed vitamin A was only present in chylomicrons. Retinyl esters were only detected in VLDLA during the postprandial period. It has not been definitely established where the retinyl esters in VLDLA come from (Krasinski et al 1990). The origin is believed to be intestinal VLDL. However, some other experiments showed that when the amount of triglycerides consumed in a meal varied, the distribution of retinyl esters in chylomicron and VLDLA varied accordingly in postprandial plasma. Thus it is more appropriate to measure retinyl esters in both lipoprotein fractions (Borel et al 1997).

To calculate the total vitamin A formed after ingestion of a test meal by each subject, we used the changes in retinyl palmitate concentration to directly infer the changes in total retinyl esters concentration. The relative distribution of retinyl esters, including palmitate, stearate, linoleate and oleate, essentially remains constant in postprandial plasma. The postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate responses were shown to be directly proportional to the postprandial TRL total retinyl ester responses (Berr and Kern, 1984).

The shape of the absorption curves of carotenoids varies among subjects and different treatment meals for one subject resulted in different absorption curves as well. As reported

before, matrix of food, molecular linkage, effectors of absorption and conversion, the nutritional status of the subject, and genetic factors all affected the bioavailability and bioconversion of β-carotene (Castenmiller and West 1998). The provitamin A carotenoids in β-carotene-biofortified cassava are in the cassava matrix. In general, the food matrix lowers the bioavailability of β-carotene at different levels, compared with the bioavailability of β-carotene dissolved in oil (De Pee et al 1998). In this theory, the matrix determines if carotenoids from biofortified cassava porridge have different bioavailability with a vitamin A or β-carotene reference dose added to white cassava porridge. The efficiency of the enzyme β-carotene 15,15' monoxygenase (BCMO1), which converts β-carotene into retinal, is affected by its genetic polymorphism. People who carry both variant alleles were found to have reduced ability in converting β-carotene into retinol (Leung et al 2009**)**. Moreover, factors from subjects themselves, such as nutritional status, also have a great impact on the absorption and conversion (Castenmiller and West 1998). When those factors, as well as the food matrix effect play a combined role on postprandial plasma total retinyl palmitate concentration, it is not hard to understand that why the shapes of absorption curves vary among subjects and test meals.

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Table 1. Reference dose concentrations

	Reference dose concentration (µg/g)	
	Analyzed by HPLC*	Calculated from label
β-Carotene content in reference dose	65.14 ± 0.64	67.20
Vitamin A content in reference dose	36.52 ± 0.96	35.70

* Values are the means of three independent analyses ± SE.

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Table 2. Carotenoid and moisture contents in the cassava porridges*

	Total carotenoid content (µg)			Moisture content	Weight of porridge (g)
	Trans β-carotene	9-C*is*-β-carotene	13-C*is*-β-carotene		
Yellow cassava porridge	970.71 ± 70.12	118.65 ± 0.69	134.93 ± 0.49	81.85 ± 0.14%	217.34 ± 5.53
White cassava porridge	34.50 ± 1.00	17.57 ± 0.26	17.28 ± 0.45	81.57 ± 0.47%	220.37 ± 1.68

* Values are the means of three replicates ± SE.

Table 3. Macronutrient composition of the porridges

	White cassava porridge				Yellow cassava porridge			
	1	2	3	Average	1	2	3	Average
Calories (Cal/serving)	142.8	143.2	142.8	142.93 ± 0.16	149.2	148.8	146.8	148.27 ± 0.91
Calories from fat (Cal/serving)	2.16	2.16	2.16	2.16	3.96	3.96	3.96	3.96
Fat	0.118%	0.104%	0.087%	0.102% ± 0.01%	0.21%	0.19%	0.18%	0.195% ± 0.008%
Total carbohydrates	15.029%	15.084%	15.066%	15.06% ± 0.02%	16.14%	16.10%	15.86%	16.04% ± 0.11%
Soluble fiber (g/serving)	<0.4	<0.4	<0.4	NA	0.432	0.512	<0.4	NA
Insoluble fiber (g/serving)	1.824	2.02	1.868	1.90 ± 0.07	2.608	2.612	2.756	2.66 ± 0.06
Total dietary fiber (g/serving)	1.824	2.02	1.868	1.90 ± 0.07	3.04	3.124	2.756	2.97 ± 0.13
Protein	0.91%	0.91%	0.91%	0.91%	0.57%	0.57%	0.57%	0.57%

Table 4. Efficiency of cyanogen removal in cassava by the wetting/spreading method

Sample No.	Cyanide content in samples (mg HCN/kg sample)			% of Cyanogen removed after 30°C for 5 h	Average	% of Cyanogen removed in final porridge	Average*
	Wet dough	After 30°C for 5h	Porridge				
1	41.20	1.98	1.07	95.19%	87.54% ± 4.90%	97.40%	93.35% ± 4.72%
2	33.25	4.75	0.99	85.71%		97.02%	
3	15.25	2.79	2.19	81.70%		85.64%	

* Values are the means of four replicates ± SE.

Table 5. Cyanide content in test porridges

Porridge		Cyanide content (mg HCN/kg porridge)	Average*
Yellow cassava	1	1.19	0.99 ± 0.14
	2	0.99	
	3	0.79	
White cassava	1	3.96	3.30 ± 1.19
	2	4.55	
	3	1.39	

* Values are the means of four replicates ± SE.

Table 6. Intra-assay precision of the BC and RP analysis in the quality control material[3]

Sample	RP peak area (nC)	RP weight (ng)	BC peak area (nC)	BC weight (ng)
1	2080	15.756	573	4.2624
2	2220	16.764	547	4.1376
3	2300	17.34	585	4.32
4	2510	18.852	618	4.4784
5	2260	17.052	566	4.2288
6	2240	16.908	580	4.296
Average		17.11		4.29
CV		5.89%		2.64%

[3] Quality control (QC) materials were aliquots from a combined chylomicron and VLDLA pool isolated from extra plasmas collected during the study.

Table 7. Inter-assay precision of the BC and RP analysis in the quality control material[4]

Sample	RP peak area (nC)	RP weight (ng)	BC peak area (nC)	BC weight (ng)
1	2320	17.484	674	4.7472
2	2250	16.98	639	4.5792
3	2560	19.212	676	4.7568
4	2140	16.188	620	4.488
5	2320	17.484	615	4.464
6	2300	17.34	593	4.3584
7	2370	17.844	638	4.5744
8	2540	19.068	676	4.7568
9	2020	15.324	573	4.2624
10	2340	17.628	603	4.4064
11	2310	17.412	588	4.3344
12	2350	17.7	598	4.3824
13	2280	17.196	682	4.7856
14	2360	17.772	521	4.0128
15	2330	17.556	543	4.1184
16	2200	16.62	619	4.4832
17	2370	17.844	635	4.56
18	2450	18.42	614	4.4592
19	2530	18.996	634	4.5552
20	2280	17.196	662	4.6896
21	2310	17.412	602	4.4016
22	2220	16.764	531	4.0608
23	2120	16.044	573	4.2624
24	2280	17.196	539	4.0992
25	2110	15.972	528	4.0464
26	2250	16.98	549	4.1472
27	2310	17.412	542	4.1136
Average		17.372		4.40
CV		5.23%		5.44%

[4] Quality control (QC) materials were aliquots from a combined chylomicron and VLDLA pool isolated from extra plasmas collected during the study.

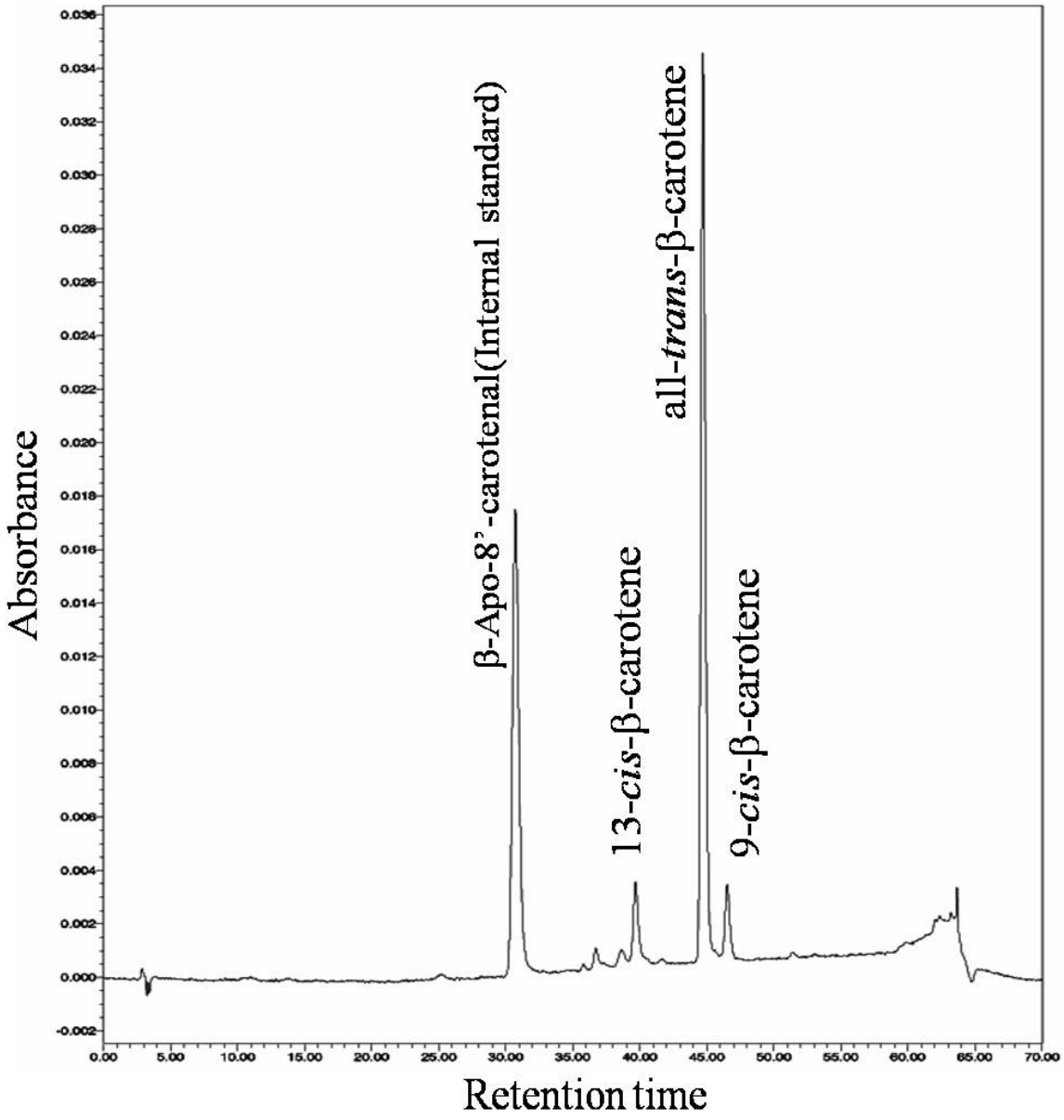

Figure 1. **Carotenoid profile of biofortified cassava porridge analyzed by HPLC UV/VIS**

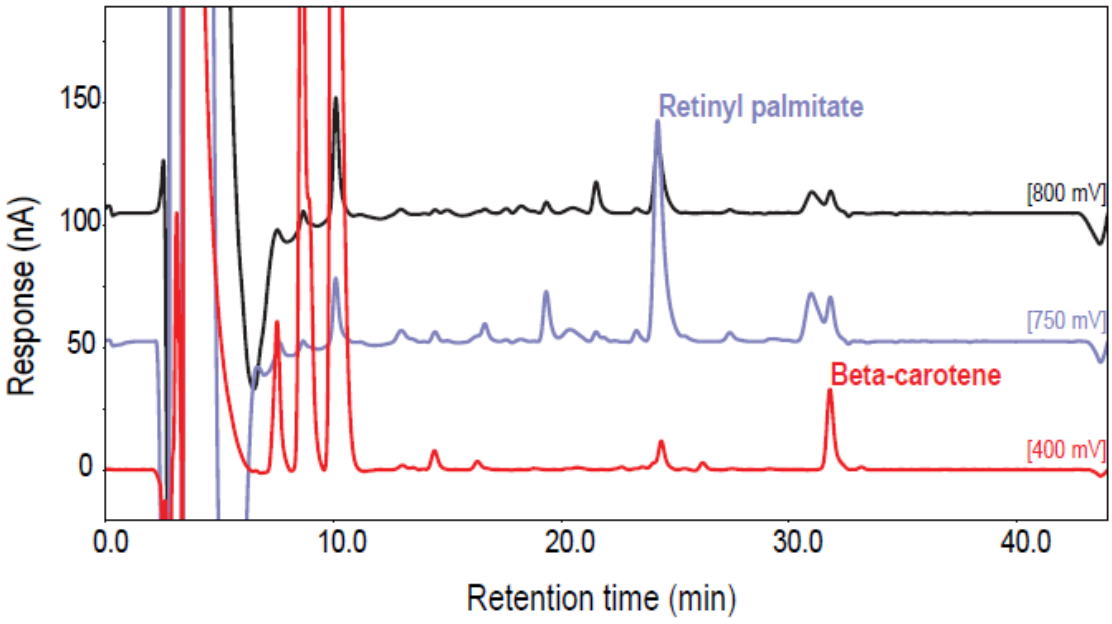

Figure 2. HPLC-ECD chromatogram of a chylomicron sample collected 5 hours after a subject ingested the β-carotene-biofortified cassava porridge

Table 8. Vitamin A equivalence values of the test porridges

Subject No.	Area under curve			Vitamin A (nmol) formed after ingestion		Vitamin A equivalence	
	Yellow Cassava ingestion	White cassava w/ BC ingestion	White cassava w/ VA ingestion	Yellow cassava	White cassava w/ BC	Yellow cassava	White cassava w/ BC
1	170.53	171.28	301.84	552.00	494.22	6.62	3.49
2	204.17	106.06	70.52	2828.94	1409.28	1.15	1.22
3	247.21	191.51	164.40	1469.11	1077.94	2.37	1.60
4	238.62	175.73	182.31	1278.82	881.55	2.75	1.96
5	321.37	194.11	228.32	1375.24	770.47	2.55	2.24
6	158.89	107.75	166.64	931.60	571.52	3.85	3.02
7	133.72	108.51	72.41	1804.37	1403.99	1.90	1.23
8	229.52	76.69	85.50	2622.91	816.16	1.25	2.11
9	211.19	100.99	NA[1]	1137.97	NA	1.66	NA
Mean ± SE	212.80 ± 18.61	136.96 ± 15.12	158.99 ± 28.82	1555.66 ± 250.28	928.14 ± 122.14	2.80 ± 0.63[2]	2.11 ± 0.29[2]

[1] The data for the ingestion of the white cassava porridge with vitamin A reference dose for this subject were lost during analysis.

[2] Not significantly different by paired t test.

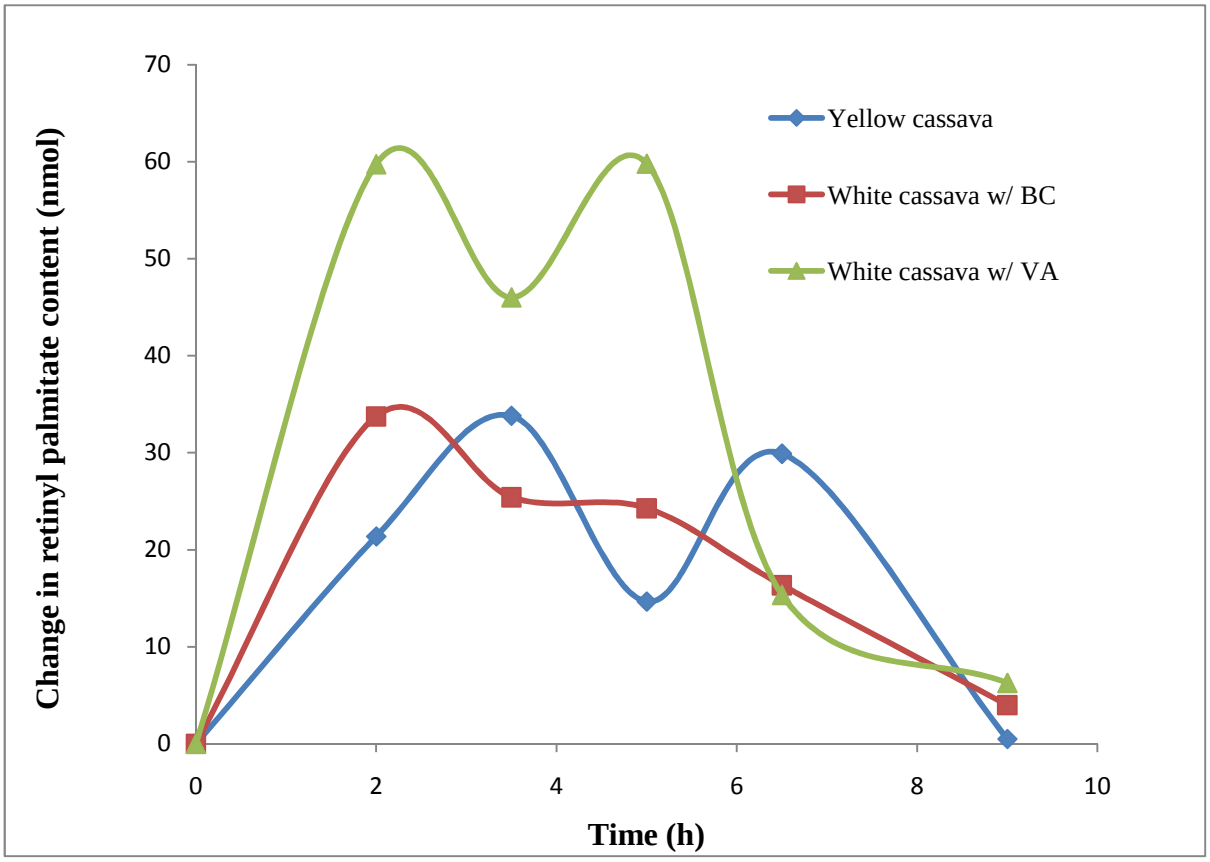

Figure 3. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 1

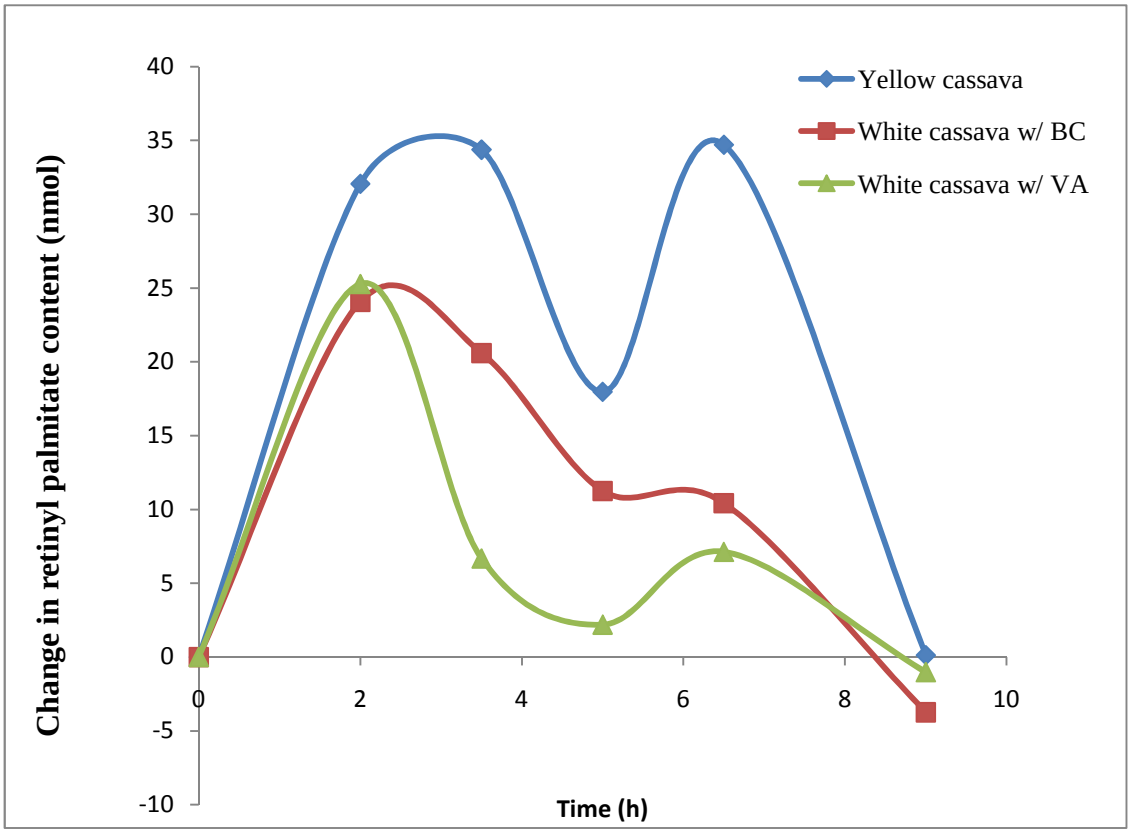

Figure 4. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 2

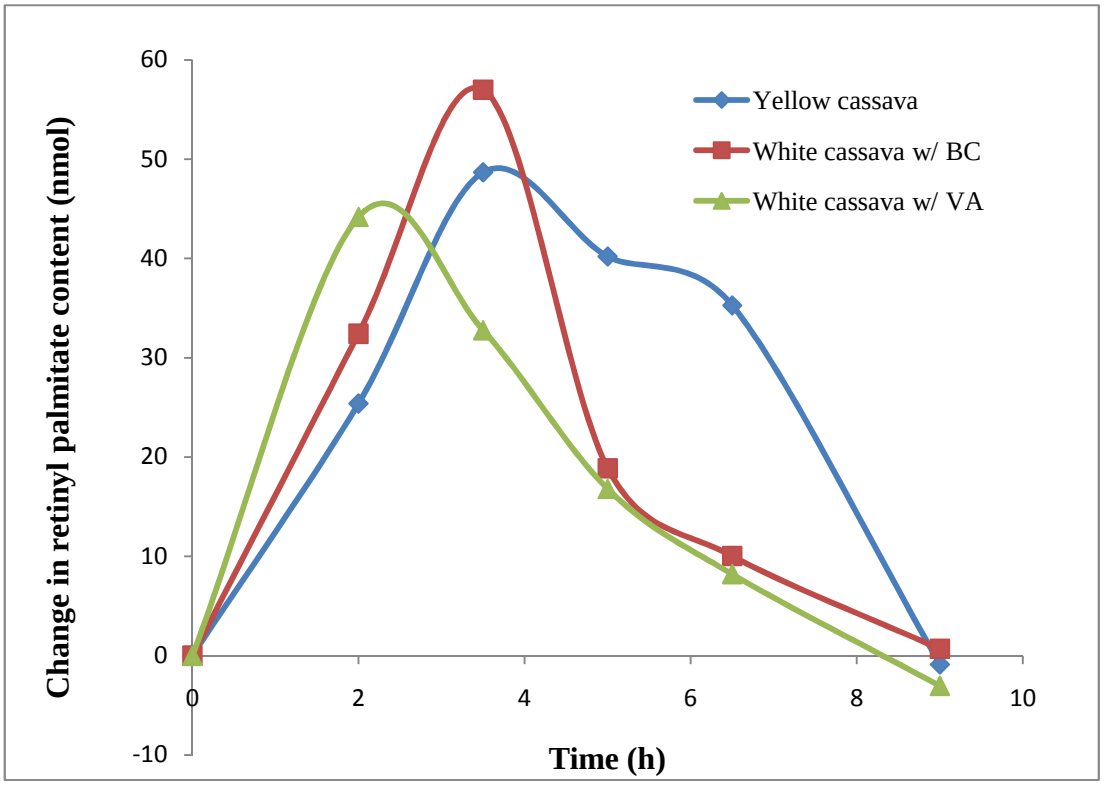

Figure 5. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 3

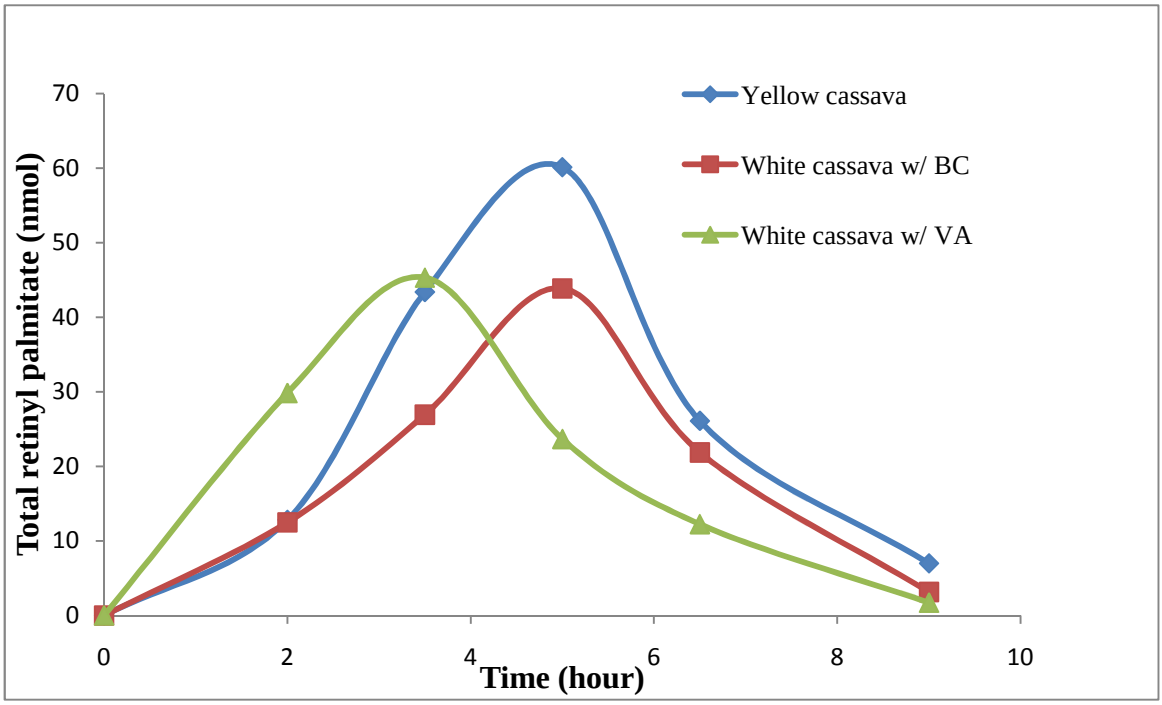

Figure 6. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 4

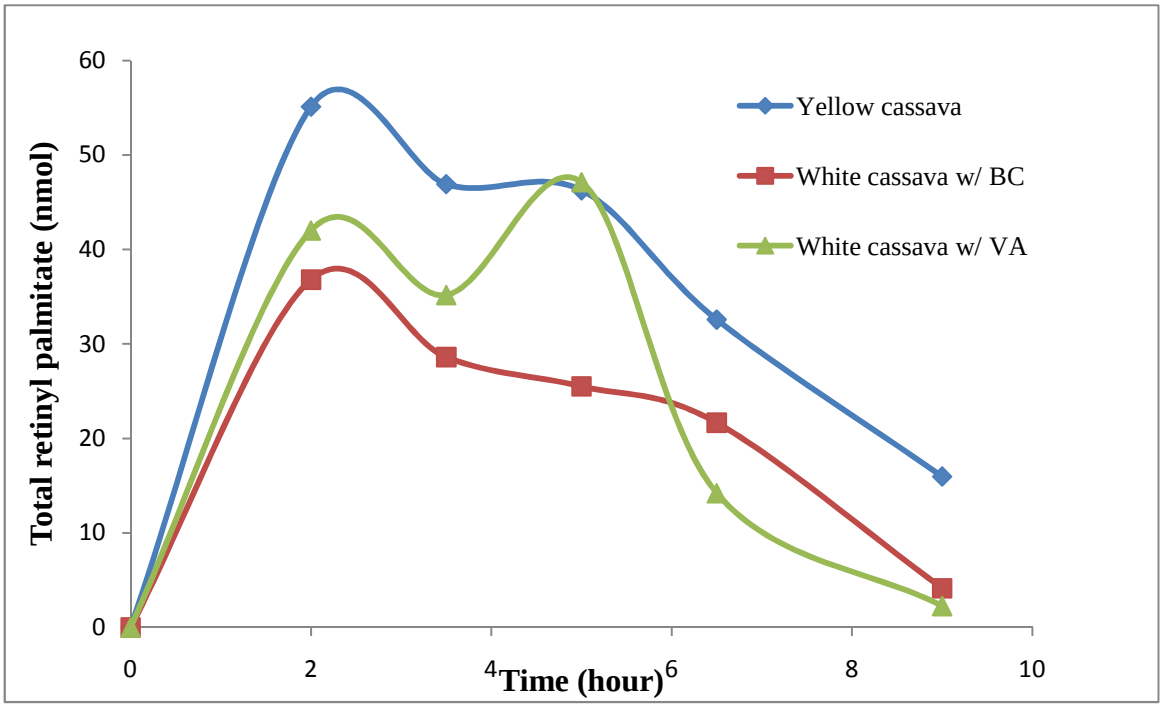

Figure 7. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 5

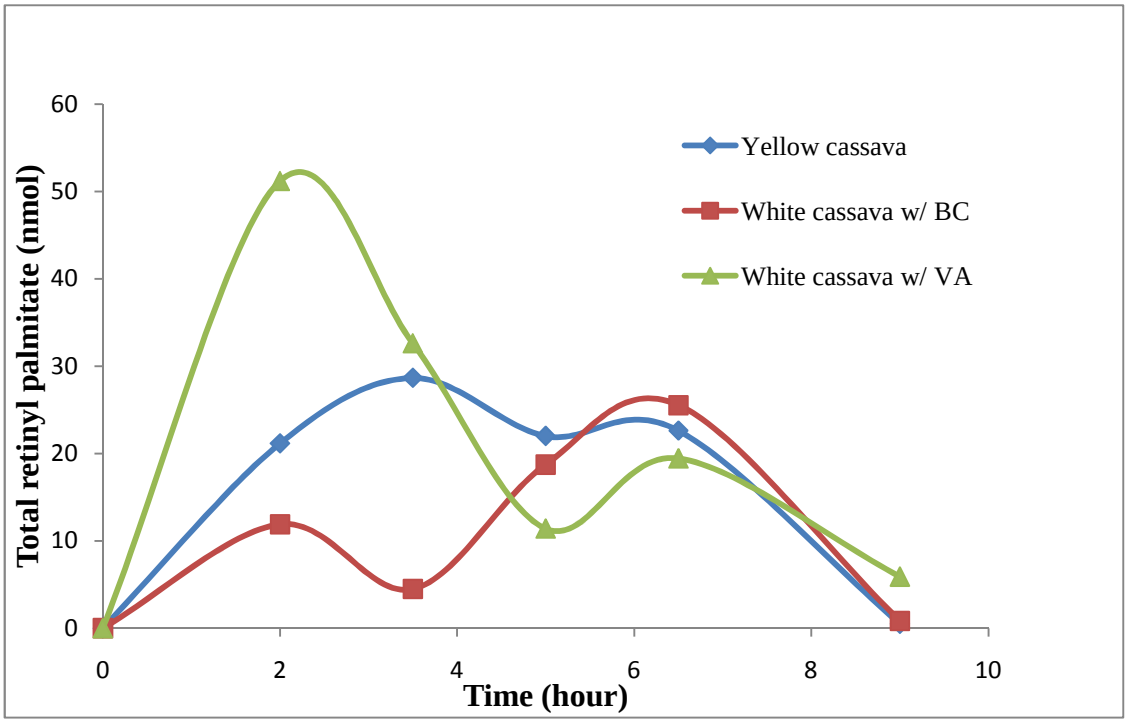

Figure 8. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 6

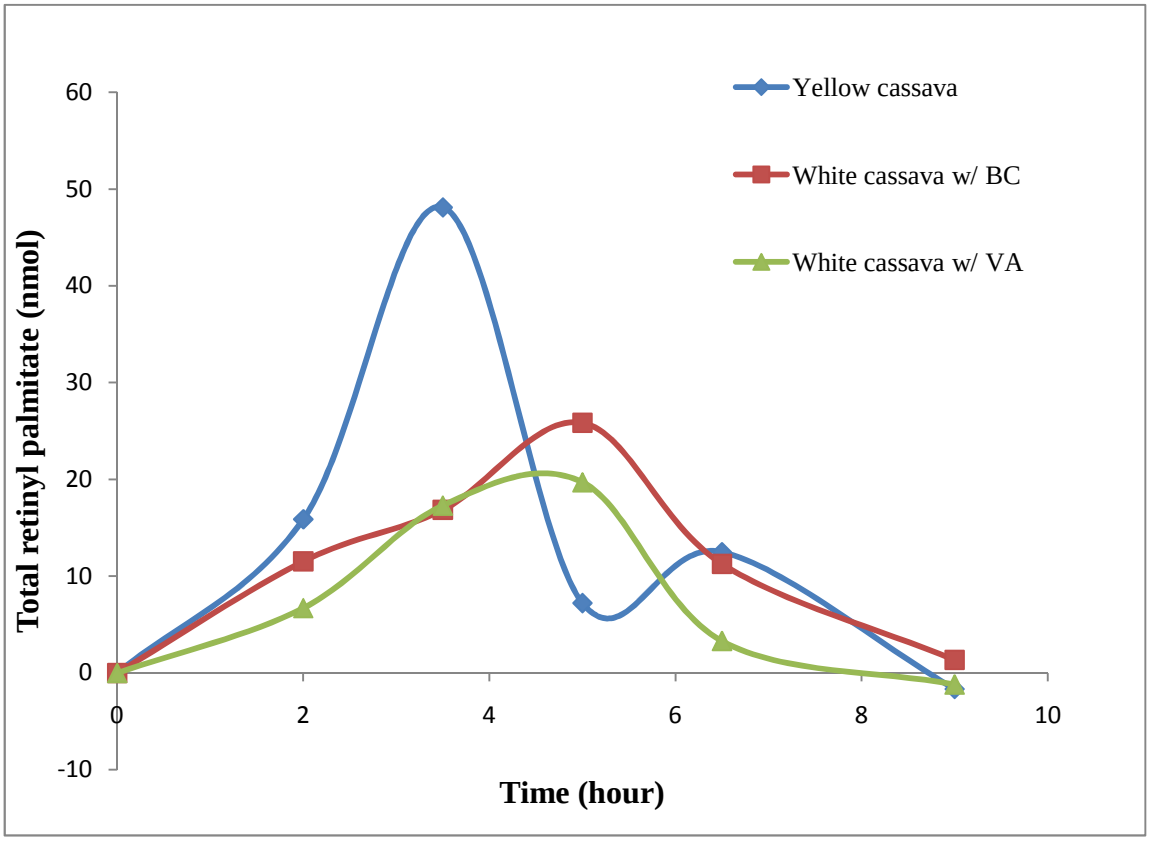

Figure 9. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 7

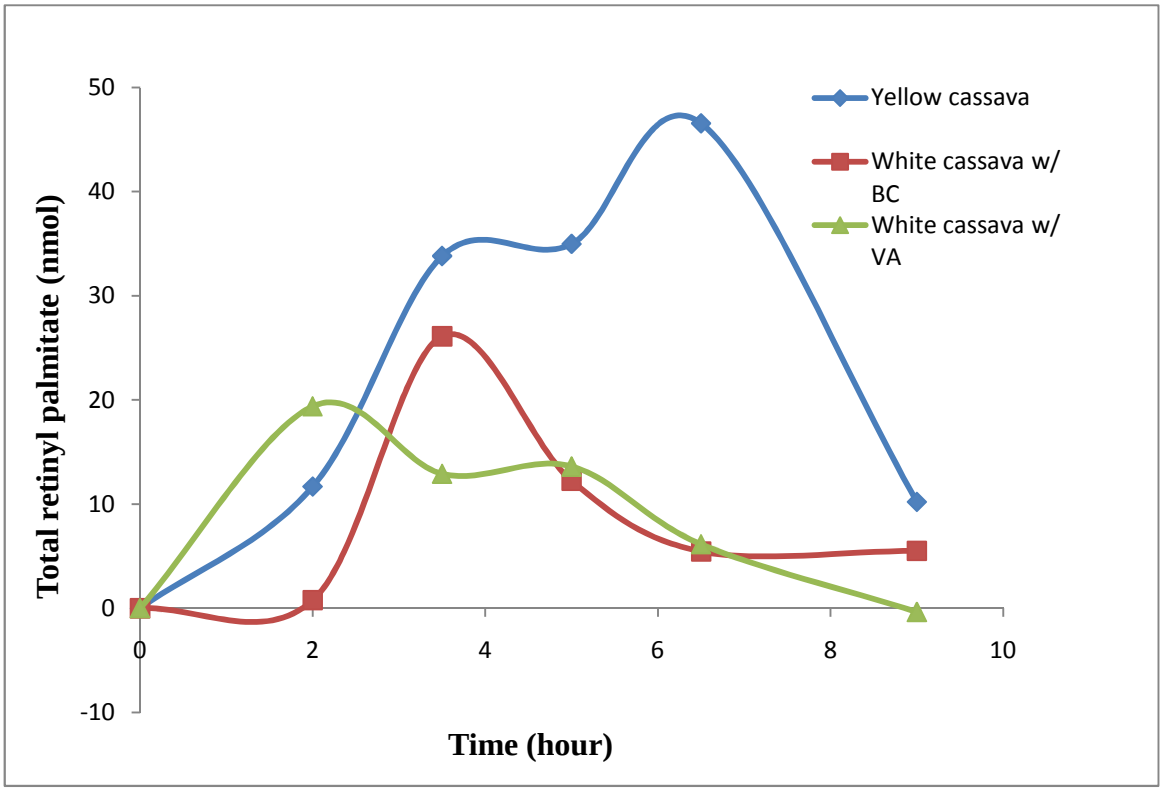

Figure 10. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 8

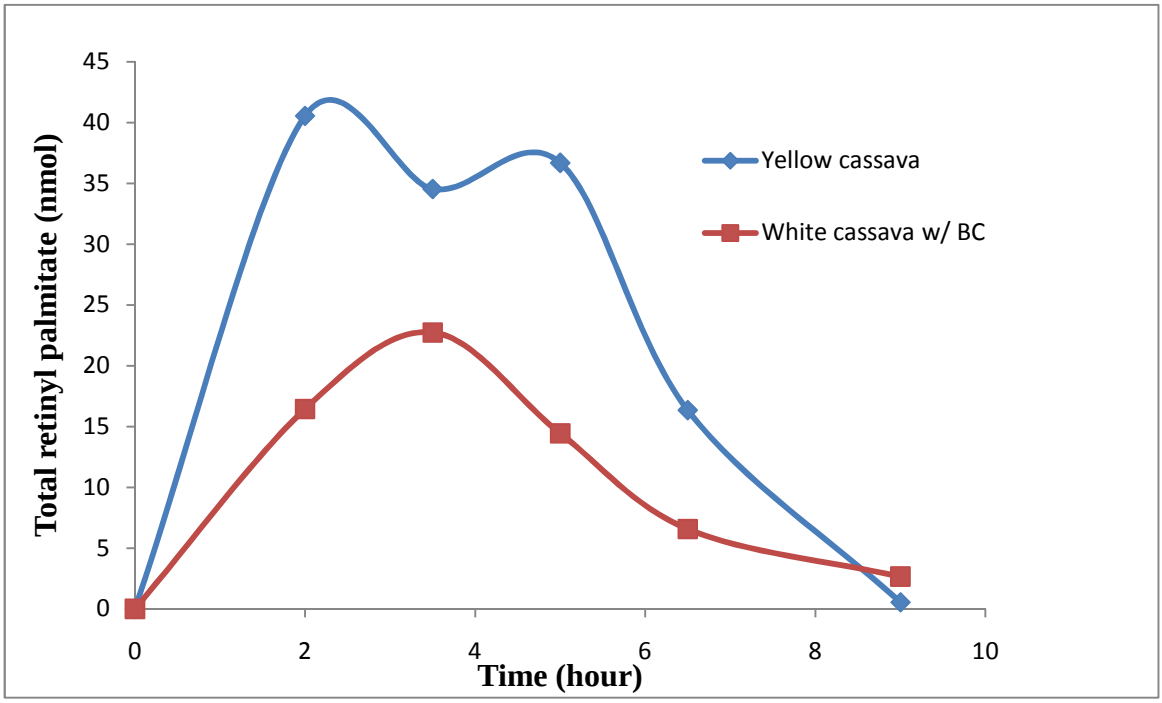

Figure 11. The appearance of retinyl palmitate in the combined chylomicron and large VLDL (VLDLA) fractions after ingestion of the test porridges by subject no. 9

CHAPTER 3. GENERAL CONCLUSIONS

The development of β-carotene-biofortified cassava targets vitamin A-deficient populations in rural areas. Before the biofortified cassava is distributed to farmers, the bioefficacy of the β-carotene in the cassava needs to be investigated.

We investigated the bioefficacy of the β-carotene in biofortified cassava in 9 healthy women ages 18-39 y. Three isomers of β-carotene were detected in the biofortified cassava porridge, including all-*trans*-β-carotene, 13-*cis*-β-carotene, and 9-*cis*-β-carotene. Before and after subjects ingested one of the three test porridges, including biofortified cassava porridge (1097.5 μg β-carotene), white cassava porridge with vitamin A reference dose (285.6 μg retinol) and white cassava porridge with β-carotene reference dose (537.6 μg β-carotene), blood samples were collected and chylomicron and large LDL (VLDLA) were separated from plasma. It was found that an average of 1587.53 ± 285.27 nmol·h of retinyl palmitate was formed in the subjects' entire plasma pool after ingestion of a serving of biofortified cassava porridge containing 1097.5 μg β-carotene. The mean vitamin A equivalence of the β-carotene was 2.80, which indicates that, after ingesting 2.80 μg β-carotene in the porridge, 1 μg of retinol will be yielded in the body. The mean vitamin A equivalence of the white cassava porridge with the β-carotene reference dose was 2.11. The vitamin A equivalence values of the β-carotene in yellow cassava porridge and white cassava porridge with the β-carotene reference dose were not significantly different by paired t test.

In conclusion, in our study population, the β-carotene in the biofortified cassava porridge had high bioefficacy, which was as good as that of a β-carotene supplement.

APPENDIX

Table 9. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.1

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	21.37781632	33.74824828	59.75506393
3.5	33.80931529	25.416963	46.02057833
5	14.66044494	24.28286134	59.79980167
6.5	29.90650668	16.34414971	15.35399172
9	0.484174171	3.982441603	6.263730714

Table 10. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.2

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	32.06160718	24.06319031	25.27891538
3.5	34.37023814	20.59364778	6.677945918
5	17.96576614	11.25358652	2.192605765
6.5	34.70004257	10.42247938	7.126479933
9	0.129942943	-3.736024504	-1.024966186

Table 11. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.3

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	25.39648492	32.42910951	44.19464207
3.5	48.66382661	56.98684104	32.78275698
5	40.19564333	18.88001626	16.81418278
6.5	35.27603209	10.04891083	8.225025451
9	-0.862948201	0.689552067	-3.025560914

Table 12. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.4

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	12.82101139	12.49732945	29.83184992
3.5	43.38540464	26.92773151	45.3144688
5	60.12066258	43.86341171	23.66886571
6.5	26.0989855	21.86706968	12.24479741
9	6.98371333	3.147631449	1.712608133

Table 13. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.5

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	55.10738825	36.80578924	42.02799689
3.5	46.9134128	28.61181379	35.18893077
5	46.26821789	25.51487819	47.12503674
6.5	32.59008565	21.66306453	14.22009597
9	15.98276848	4.146022537	2.283990006

Table 14. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.6

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	21.16042154	11.88860864	51.19228704
3.5	28.65566462	4.485614704	32.62714649
5	22.00603871	18.73042027	11.40984301
6.5	22.62103301	25.53379475	19.4432061
9	0.500456643	0.841773453	5.913331419

Table 15. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.7

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	15.8857799	11.53772879	6.70934076
3.5	48.10262716	16.86155483	17.30529452
5	7.224552925	25.859275	19.70761602
6.5	12.49679065	11.27375161	3.311771782
9	-1.651167045	1.351305844	-1.181856381

Table 16. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.8

Time	Total retinyl palmitate (nmol)		
	Yellow cassava	White cassava w/ BC	White cassava w/ VA
0	0	0	0
2	11.6909319	0.769862824	19.38488128
3.5	33.81950456	26.11113395	12.90067237
5	34.98085542	12.23821848	13.60232184
6.5	46.5459743	5.458832894	6.135803661
9	10.21504852	5.516900437	-0.362922141

Table 17. Total postprandial plasma triacylglycerol-rich lipoprotein (TRL) retinyl palmitate of subject No.9

	Total retinyl palmitate (nmol)	
Time	Yellow cassava	White cassava w/ BC
0	0	0
2	40.54329357	16.43849574
3.5	34.54184245	22.73189169
5	36.68722438	14.43770512
6.5	16.35191607	6.572779077
9	0.552945772	2.654867262

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