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Quantifying the bioefficacy of β-carotene-biofortified sorghum

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Quantifying the bioefficacy of β-carotene-biofortified sorghum

by

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ABSTRACT

Vitamin A (VA) is an essential fat-soluble nutrient needed by all vertebrates and plays a central role in essential physiological functions including normal growth, development, immune function, epithelial integrity, reproduction, and vision. Vitamin A deficiency (VAD) is a significant public health problem in more than half of countries worldwide. VAD is a major cause of preventable childhood blindness and also impairs immune function, especially affecting the poorest populations in low and middle-income countries. Based on the World Health Organization (WHO) threshold definition for biochemical VAD (serum retinol below 0.7 µmol/L), most countries in Sub-Saharan Africa and East Asia have over 20% of preschool-age children suffering from VAD. Biofortification is the technique of using plant breeding and/or biotechnology to produce micronutrient-enhanced staple food crops. Because most populations in these areas heavily rely on plant-based diets, provitamin A biofortification is a promising approach to alleviate widespread VAD.

Sorghum is the fourth most important cereal crop worldwide and in Africa. Approximately 300 million of Africa's most food insecure people who live in the semi-arid tropics rely on sorghum as their staple crop. To alleviate the prevalence of VAD in these areas, the Africa Biofortified Sorghum (ABS) initiative was founded to develop β-carotene-biofortified sorghum through genetic engineering. Our objective was to quantify the bioefficacy of the biofortified sorghum using a Mongolian gerbil model.

Gerbils were fed a VA-free control diet (45% by wt non-transgenic sorghum flour) for 4 wk; 8 animals were then killed at baseline. The remaining animals were randomly

assigned to treatments for 6 wk (n = 11 per group): 1) control diet dosed daily with cottonseed oil vehicle; 2) biofortified diet (45% by wt transgenic sorghum flour) (99.6 nmol retinol equivalents/d) dosed daily with cottonseed oil vehicle; 3) control diet dosed daily with β-carotene (84.9 nmol retinol equivalents/d); 4) control diet dosed daily with VA (85.1 nmol retinol equivalents/d). Liver VA stores in the baseline (1.27 ± 0.29 µmol), β-carotene-dosed (1.30 ± 0.26 µmol), and biofortified (1.31 ± 0.23 µmol) groups were not different; liver VA stores in the vehicle-dosed control group were lower (0.92 ± 0.22 µmol) ($P < 0.01$). Liver VA stores were highest in the VA-dosed group (2.48 ± 0.23 µmol) ($P < 0.0001$). The calculated bioconversion efficiency for the β-carotene in the biofortified sorghum (4.5 µg β-carotene to 1 µg retinol) was similar to that of the β-carotene dose (3.8 µg to 1 µg retinol). The β-carotene and the other provitamin A carotenoids in the biofortified sorghum: 1) effectively restored liver VA stores in a VA-depleted animal model; 2) had efficacy similar to that of a supplemental β-carotene dose in maintaining liver VA stores.

Accurate quantification of hepatic retinol and retinyl esters is necessary for the determination of vitamin A status in animal models. Our objective for the second study was to optimize methods for the extraction and HPLC analysis of hepatic retinol and retinyl esters.

Our results indicated that Method #1 (J Lipid Res 2014;55:1077-86) resulted in higher hepatic total vitamin A concentrations (423 ± 72.9 nmol/g) than Method #2 (Methods Mol Biol 2010;652:263-75) (36.8 ± 5.35 nmol/g), Method #3 (Anal Methods 2010;2:1320-1332) (347 ± 26.6 nmol/g), and Method #4 (Food Chem 2014;159:477-85) (288 ± 49.1 nmol/g) ($P < 0.0001$). Of the many factors evaluated within the hepatic VA extraction protocol, an adequate concentration of ethanol (at least 50%) in the homogenization media is

necessary for exhaustive extraction. Ethanol is needed in the homogenization media to extract retinoids from liver tissues and then partition them directly to hexane. Homogenizing liver in 100% PBS might facilitate liver tissue binding with water molecules and therefore interfere with the above partitioning process. The initial extraction of VA from the tissue into ethanol is a critical step that needs to be facilitated by an adequate duration of vortexing. Finally, the selection of the reconstitution solvent is important for optimal chromatography and therefore for accurate quantification.

CHAPTER 1

INTRODUCTION

Vitamin A is an essential nutrient for many physiological functions (1). The carotenoids containing the VA structure are called provitamin A carotenoids and have the potential to be bio-converted into vitamin A in humans and other vertebrates (2). Vitamin A deficiency (VAD) is a prevalent malnutrition disease affecting the poorest populations in low and middle-income countries (3-5). Based on the World Health Organization (WHO) threshold definition for biochemical VAD (serum retinol below 0.7 µmol/L), most countries in Sub-Saharan Africa and East Asia have over 20% of preschool-age children and over 10% of pregnant women suffering from VAD (6). VAD accounts for as high as 2% of all deaths in children younger than five years in both sub-Saharan Africa and South Asia (7).

The estimated prevalence of VAD in children ages 6-59 months has declined from 39% (data from 1991) to 29% (data from 2013) in low and middle-income countries in the last decades. However, this trend of reduction should only be attributed to the significantly decreased prevalence of VAD in Oceania, Latin America, the Caribbean region, and East and Southeast Asia. No evidence of a reduction in the estimated prevalence of children with VAD can be found in sub-Saharan Africa region from the year of 1991 (estimated prevalence 45%) to the year of 2003 (estimated prevalence 48%).

People in sub-Saharan Africa have limited access to the foods that contain high levels of VA (e.g. meat, eggs and dairy products) or provitamin A carotenoids (carrot, pumpkin, orange-fleshed sweet potato, and green leafy vegetables) (8, 9). Staple foods (maize, rice, sorghum, and wheat) of population susceptible to VAD are typically low in provitamin A (10).

Because most populations in these areas heavily rely on plant-based diets, provitamin A biofortification is believed as a promising approach to alleviate widespread VAD (8).

Provitamin A biofortification approaches (11) have been successfully applied in multiple crops such as cassava (12), maize (13), rice (14), and sweet potato (15) by means of transgenic and/or conventional breeding. According to the most updated Food and Agricultural Organization (FAO) data, sorghum ranks as the fourth most important cereal crop in Africa in terms of production in tonnes (16). Approximately 300 million of African rely on sorghum as their staple diet (17). However, conventional sorghum lines have nutritional flaws. The limitations include low concentrations of provitamin A carotenoids and tocopherols (vitamin E), poor iron and zinc bioavailability due to anti-nutrients such as phytate and tannins, and low protein digestibility due to the cross-linking of sorghum kafirin proteins through their disulfide bonding. The Africa Biofortified Sorghum (ABS) initiative was therefore founded in 2005 to address these nutritional limitaitions through genetic engineering (18).

In this study, our objective was to quantify the bioefficacy of the biofortified sorghum (ABS 203) using a Mongolian gerbil model because of its similarity with human in metabolizing carotenoids. With the completion of several method development studies, we were able to accurately identify and quantify the provitamin A carotenoids in the biofortified sorghum. The bioefficacy (VA equivalence value) of provitamin A carotenoids in the biofortified sorghum was then determined.

In mammals, the liver is the major vitamin A (VA) storage site that contains 50% to 80% of the body's total VA (retinol plus retinyl esters) (19). Accurate quantification of hepatic VA is necessary for the determination of VA status in animal models. There are many published hepatic VA extraction methods, and their details can vary considerably from

each other. To optimize the hepatic VA extraction method and understand the rationale underlying each extraction step, we compared in rodent livers published non-saponification extraction protocols for HPLC analysis of retinol (ROL) and retinyl esters. We then investigated the effects of several key factors in hepatic VA extraction efficiency, including the amount of ethanol added before homogenization of the liver tissue and the duration of vortexing before adding hexane. The selection of reconstitution solvent was also investigated for optimal chromatography and therefore for accurate quantification.

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CHAPTER 2

LITERATURE REVIEW

Physiological Functions of Vitamin A in Humans

What is vitamin A?

Vitamin A (VA) is an essential fat-soluble nutrient needed by all vertebrates for normal growth, development, immune function, epithelial integrity, reproduction, and vision (1-3). The term VA is used to refer to compounds that possess the biological activity of retinol. Preformed VA (retinol and its esterified form, retinyl esters) and provitamin A carotenoids (mostly β-carotene) are the main forms of VA in natural foods. The major form of dietary preformed VA is long-chain fatty acid esters of retinol (i.e., retinyl esters) that can be found in animal origin foods such as dairy products, poultry, fish and meat (4). Carotenoids are synthesized by numerous plants, fungi and bacteria and thus can be found in many vegetables and fruits (5). Because the VA molecule has an unsubstituted β-ionone ring and a conjugated polyene chain with a particular number and positions of methyl groups, the carotenoids containing the VA structure are called provitamin A carotenoids and have the potential to be bio-converted into vitamin A in humans and other vertebrates (6).

Digestion, absorption, bio-conversion, and metabolism of vitamin A

Preformed VA is highly bioavailable. Prior to intestinal absorption, retinyl esters must be hydrolyzed into free retinol by pancreatic retinyl ester hydrolase (REH) in the intestinal lumen (7). Two mechanisms have been proposed for the uptake of retinol into duodenal and jejunal mucosal cells. Unesterified retinol is known to be absorbed into

enterocytes via passive diffusion after being incorporated into mixed micelles (8). However, more recently a protein-mediated facilitated diffusion system was also shown to mediate the direct absorption of free retinol (9). Cellular retinol-binding protein II (CRBP II) is one of the most abundant proteins in intestinal mucosal cells. CRBP II is a transporter that is uniquely adapted to the intestinal absorption and metabolism of VA (10). Under postprandial conditions, retinol is absorbed rapidly by intestinal cells. Retinol is then esterified and secreted by mucosal cells in the form of retinyl esters. Under fasting conditions, some unesterified retinol is directly secreted from the mucosal cells into the basolateral medium (11).

Among provitamin A carotenoids, β-carotene has the greatest provitamin A activity because symmetrical cleavage of β-carotene in the digestive system provides two retinal (retinaldehyde) molecules. In contrast, other provitamin A carotenoids such as α-carotene and β-cryptoxanthin provide only one retinal molecule due to the presence of only one β-ionone ring (12). Because dietary carotenoids are often embedded in food matrices and complexed with proteins and fatty acids, some digestion is required before carotenoids can be made available to be absorbed in the small intestine. After being released from food matrices by mechanical and enzymatic disruption in the digestive system, carotenoids are hydrolyzed from proteins by pepsin in the stomach and proteolytic enzymes in the duodenum. The hydrolysis of carotenoid esters is catalyzed by various hydrolases and esterases of pancreatic origin (13).

Like VA, early studies indicated that the intestinal absorption of carotenoids occurred by passive diffusion. Free carotenoids were believed to be incorporated into micelles with other lipids and absorbed via passive diffusion across the brush border membrane of

enterocytes. However, there is recent evidence that the absorption of carotenoids is saturable and protein facilitated (14, 15). The facilitating receptor for the uptake of carotenoids was identified as scavenger receptor class B type 1 (SR-B1), the mammalian counterpart of *Drosophila* NinaD (14). The bio-conversion of provitamin A carotenoids to VA takes place in the small intestinal mucosal cells. There are two mechanisms that have been proposed for the cleavage of carotenoids. The major pathway is the central cleavage of provitamin A carotenoids (especially β-carotene) to retinal (VA), which is catalyzed by β-carotene 15,15'-oxygenase 1 (BCO1) (16, 17). In contrast, β-carotene 9',10'-oxygenase 2 (BCO2) catalyzes the eccentric cleavage of carotenoids, yielding long-chain apocarotenoids (18, 19). Compared with BCO1 that is primarily involved in provitamin A carotenoid bio-conversion, BCO2 also metabolizes non-provitamin A xanthophyll carotenoids such as lutein and zeaxanthin. A negative feedback system controls the expression of SR-B1 and BCO1 involving the gut-specific homeodomain transcription factor (ISX) (20). This feedback system tightly regulates the absorption and bio-conversion of dietary VA for animals with varying VA status. The retinol from dietary performed VA and provitamin A carotenoids is esterified and undergoes the same fate in terms of metabolism.

Newly formed retinyl esters leave the intestine in chylomicrons that are large lipoproteins incorporating triacylglycerol, phospholipids, cholesterol esters, and other fat soluble components. A portion of un-oxidized carotenoids may also leave the intestine intact as a component in chylomicrons or large very low density lipoproteins (VLDLA) (21-23). After chylomicrons enter the lymph and then the venous circulation, the triacylglycerols are partially hydrolyzed on the surface of peripheral tissue cells containing lipoprotein lipase. The remnant particles (chylomicron remnants) retaining almost all retinyl esters and a

substantial proportion of the intact carotenoids are taken up by the liver via the hepatic receptors for apolipoprotein E or B/E (24-26).

The liver plays an essential role in VA storage, metabolism and homeostasis (11, 27). The retinyl esters are rapidly hydrolyzed into retinol after chylomicron remnants are taken up by the liver. After binding with liver cellular retinol binding protein type 1 (CRBP-1), a portion of retinol is re-esterified into retinyl esters by lecithin retinol acyltransferase (LRAT) for further storage in hepatic stellate cells. Unesterified retinol can also undergo secretion by binding to retinol-binding protein (RBP) to form a complex named holo-RBP. After reaching the liver, carotenoids undergo three fates: cleavage into retinoids or apocarotenoids, incorporation into lipoproteins and secretion into the bloodstream, or storage in the liver.

In the venous circulatory system, the retinol in holo-RBP is transported to organs throughout the body and taken up by cells that possess the transmembrane protein Stra6 (a cellular RBP receptor) (28-30). Plasma retinol concentrations remain constant even though liver VA concentrations may vary within a wide range, from approximately 20 ug/g liver to 500 ug/g liver. Plasma retinol only decreases when liver VA stores are nearly depleted (31). Carotenoids are transported in the blood as part of very-low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), and high-density lipoproteins (HDLs). Uptake of carotenoids into target tissues is mediated by the cellular uptake of lipoproteins through apolipoprotein receptors on the surface of the cells. In addition to the liver, carotenoids can be stored in adipose tissues and are found in other specific tissues such as the retina of the eye (32).

Retinoic acid is the active hormonal form of VA that is oxidized from retinal by retinal dehydrogenase. After being delivered by cellular retinoic acid-binding proteins

(CRABPs) to the cell nucleus, retinoic acid binds to the retinoic acid receptor-retinoid X receptor (RAR-RXR) heterodimer on DNA to regulate gene expression (33, 34).

Physiological functions of vitamin A

VA is one of the fat soluble nutrients earliest to be discovered. Two distinct roles of VA have been convincingly shown, as: 11-cis retinal binds to opsin protein in the retina for the photo-transduction mediation needed for normal vision; and all-trans retinoic acid or 9-cis retinoic acid activates the ligand-dependent transcription factors RAR and RXR to regulate gene transcription and cell differentiation (35, 36).

Vision

VA (11-cis retinal) is needed to bind opsin and form rhodopsin in rod photoreceptor cells in the retina (37, 38). Within the photoreceptor rod cells, rhodopsin is necessary for detecting small amounts of light in a dark environment. Rhodopsin is cleaved, opsin is released, and 11-cis retinal is isomerized to all-trans retinal when a photon of light hits the retina. In response to the conformational change (photoisomerization), a complex signal transduction cascade occurs, and an electrical signal is generated and then sent to the brain for the sense of vision (39).

Immune function

A positive relationship between VA deficiency (VAD) and increased susceptibility to natural infections has been established in animals and humans (40). As retinoic acid plays important roles in gene expression, many aspects of immunity can be influenced by VA

status. Cell differentiation and maturation of immune cells such as T/B lymphocytes, neutrophils, and macrophages need to be triggered and regulated by retinoic acid (41, 42). Also, the expression and production of cytokines and immunoglobulins require the participation of retinoic acid. Both innate and mucosal immunity will be impaired if the VA intake is inadequate (43).

<u>Growth and development</u>

VA plays a critical role in mammalian growth and embryonic development (7, 44). When the VA stores of animals are exhausted by consuming VA free diets, cessation of growth can be observed and growth resumes after subsequent VA supplementation (45-47). Vitamin A deficiency (VAD) in critical periods of development also leads to organ (heart, eye, lung, gonad, ear, and other organs) and limb malformations (37, 48). One reason is that retinoic acid regulates expression of genes related to the expression of growth hormones (7). Epidemiological studies suggest that VAD rate is positively related to stunting in children ($P < 0.001$) (49). Moreover, multiple clinical trials separately indicate that linear growth in children with VAD in Southeast Asia can be improved by VA supplementation (50-52).

Vitamin A Deficiency in Africa

Dietary requirements for vitamin A

The U.S. Academy of Medicine has adopted retinol activity equivalents (RAE) as the measurement unit for recommended VA intakes (53). The RAE is used to account for the differences in the bioactivities of various preformed VA and provitamin A carotenoids and for the different dietary matrices. The Recommended Dietary Allowance (RDA) for VA is

the average daily dietary intake that is sufficient to meet the nutritional requirement for almost all (97-98%) healthy individuals. The RDAs for VA for adult men, adult women, adult pregnant women, adult lactating women, children ages 1-3 y, and children ages 4-8 y are 900 μg RAE, 700 μg RAE, 770 μg RAE, 1300 μg RAE, 300 μg RAE, and 400 μg RAE, respectively.

The clinical signs of inadequate VA intake have been well studied. Multiple biomarkers have been used to determine the sufficiency status of VA. Xerophthalmia caused by severe VAD is an eye disease with destructive dryness of the conjunctival epithelium (54, 55). Night blindness results from marginal VAD when the serum retinol concentration is 0.35 to 0.70 μmol/L and the VA pool in the rod cells of the retina is inadequate. Due to the effect of VA on immune function, VAD is also associated with increased risk for morbidity and mortality from diarrhea and measles (56). The concentration of serum retinol is tightly controlled and, therefore, inaccurate to directly reflect the liver VA stores (VA status) of an individual. However, when liver VA concentration falls below a critical cutoff (20 μg/g liver), serum retinol concentration declines (57). Because of its feasibility of measurement, serum retinol concentration is the most common indicator used to determine the prevalence of VAD in large populations.

Preformed VA and provitamin A carotenoids are major VA sources for human populations. In developed countries, approximately 66% to 80% of dietary VA intake is contributed by preformed VA from animal-derived foods such as butter, cheese, and egg (Western dietary patterns), and 20% to 34% of VA intake is from provitamin A carotenoids. In contrast, for most people living in developing countries, only about 12% to 22% of dietary VA comes from preformed VA and 78% to 88% comes from provitamin A carotenoids. The

majority of the populations in developing countries have dietary patterns that are high in plants (staple grains and vegetables) and low in meats and dairy products.

Prevalence of vitamin A deficiency in Africa

As one of the most widespread micronutrient malnutrition diseases worldwide, VAD is a major cause of preventable childhood blindness and also impairs immune function, especially affecting the poorest populations in low and middle-income countries (40, 52, 58). VAD accounts for as high as 2.0% of all deaths in children younger than five years in both sub-Saharan Africa and South Asia (59).

Based on the World Health Organization (WHO) cutoff definition for biochemical VAD (serum retinol below 0.7 µmol/L), most countries in sub-Saharan Africa have over 20% of preschool-age children suffering from VAD (56). In the past decades, the estimated prevalence of VAD in children ages 6-59 months has decreased from 39% (data from 1991) to 29% (data from 2013) in low and middle-income countries due to reasons such as VA supplementation programs and improvement of overall nutrition (59). However, this decline should only be attributed to the significantly decreased prevalence of VAD in East and Southeast Asia, Oceania, Latin America, and the Caribbean. No evidence of a reduction in the estimated prevalence of children with VAD was observed in sub-Saharan Africa between 1991 (estimated prevalence 45%) and 2003 (estimated prevalence 48%).

Globally, about 19.1 million pregnant women have low serum retinol concentration (< 0.7 µmol/L), and approximately half of them suffer from moderate to severe VAD that can lead to gestational night blindness. Nearly all African countries have over 10% of pregnant women in biochemical VAD (56). VAD women are likely to give birth to children who are

at high risk for VAD (60). Semba et al. (61) found a three-fold higher likelihood ($P < 0.03$)
of mortality in infants who were born to women with low serum retinol concentrations (<
0.32 μmol/L). A cross-sectional epidemiological study also showed a positive relationship
between maternal VAD and increased risk of maternal anemia and preterm delivery in India
(62). In addition, gestational night blindness was positively associated with higher risk of
early mortality (death before 6 months of life) in Nepalese infants (63). If these results can
be extrapolated to African, the control of VAD in child bearing-age women is of importance
to the population health of both women and children in Africa countries.

Vitamin A Biofortification of Sorghum

Strategies to alleviate vitamin A deficiency

VA-rich foods are not always accessible to people. Various strategies have been
adopted by different countries and organizations to alleviate widespread VAD and its impacts
on public health. Because VAD often occurs in clusters, VA supplementation, and food VA
fortification are the most common strategies that are pursued by low and middle-income
countries.

Vitamin A supplementation

VA supplementation is proven to be a successful and efficient approach to alleviate
VAD. Millennium Development Goal 4 (MDG 4) focused on improving child survival by
reducing by two-thirds the mortality rate among children by 2015 (64). VA supplementation
was an important part of MDG 4 and is recognized as one of the most cost-effective
interventions for improving child survival (65). Generally, a VA dose in the form of retinyl

esters is first dissolved in oil and then contained in soft gelatin capsules. Infants 6 to 11 month of age are given a single VA supplement in a dose of 30 mg RAE. Children 12 to 59 months of age are given a VA supplement in a dose of 60 mg RAE every 4-6 months (66). A number of clinical trials reported a significant reduction in mortality in children following VA supplementation (67, 68). Globally, approximately 75% of children aged 6 to 59 months received two doses of VA supplement in 2011, resulting in protection against VAD (69). Unfortunately, VA supplementation is only comprehensively successful in urban areas or developed countries because of the effectiveness of the project execution. Many children in remote areas in low income countries are not able to be reached by supplementation programs. Besides the cost of the VA capsules, millions of dollars are needed to operate the programs and distribute the capsules every year. Therefore, VA supplementation programs are often implemented by non-profit organizations in many African countries and are not economically sustainable because the funding is not always guaranteed (65).

Vitamin A fortification

Adequate intake of foods containing enough of a specific nutrient is fundamental to control nutrient deficiency on a sustainable basis. Food fortification has many advantages over other approaches: it is socially acceptable; it has low costs of operation; it only requires minimal changes in eating habits; and it is economically sustainable because it shares the same logistics system with traditional foods (70). Food fortification is a major contributor to nutrient intakes for both developed and developing countries (71, 72).

People in "high-risk" regions for VAD have limited access to the foods containing high levels of preformed VA (e.g. meat, eggs and dairy products) or provitamin A

carotenoids (carrots, pumpkin, sweet potatoes, and green leafy vegetables) (73, 74). Staple foods (maize, rice, sorghum, and wheat) of populations susceptible to VAD are typically low in provitamin A (75). Margarine has long been successfully fortified with VA in Western countries (76, 77). In an intervention study in the Philippines, VA-fortified margarine substantially decreased the prevalence of low serum retinol in preschool age children from 25.7% to 10.1% after 6 months of consumption (78). Sugar is a popular vehicle for VA fortification (79). VA compound (retinyl palmitate) is bound to the sugar crystal as a gum-based beadlet with a layer of vegetable oil to avoid segregation (80). In Central America countries such as Guatemala, VA has been added to sugar since 1974 (81). Cooking oil is another ideal matrix for VA fortification because VA is fat-soluble. In Brazil, VA fortified cooking oil significantly improved plasma retinol concentrations and liver VA stores (82).

Provitamin A biofortification is a relatively new concept. Conventional cross-breeding techniques were used to transfer micronutrient density traits into high-yielding competitive genetic backgrounds (83). For example, while typical maize has β-carotene concentrations of only 0.07 to 1.4 μg/g (84), some varieties of biofortified maize have about 10 μg β-carotene per g (85). Human and animal bioefficacy studies indicate that biofortified maize is a good source of vitamin A with high vitamin A bioavailability (74, 86, 87). "Yellow" cassava containing high concentrations of β-carotene has also been produced through conventional plant breeding. Our laboratory and another research group have shown high β-carotene bioefficacy values from biofortified cassava (88, 89).

Transgenic biofortification is also a promising approach to enhance the provitamin A value of traditional staple foods. Unlike conventional breeding, genetic engineering can increase the micronutrient concentration in the crop that does not naturally contain a

micronutrient. The transgenic approach also facilitates the incorporation of multiple genetic systems from different organisms (animals, plants, and microorganisms) to simultaneously increase micronutrient concentrations, decrease anti-nutrient concentrations, and enhance promotors of bioavailability (90).

Among several transgenic biofortification approaches for staple crops, the "Golden Rice" project is perhaps the most famous (91). The maize gene encoding phytoene synthase (PSY) and the Erwinia uredovora gene encoding carotene desaturases (CRTI) were expressed in rice endosperm through *Agrobacterium tumefacien*-mediated transformation. The advanced version of Golden Rice provides up to 37 µg/g total carotenoids (of which 84% is β-carotene) (92, 93). A clinical trial (94) suggested that the β-carotene in Golden Rice has high bioefficacy (3.8 µg β-carotene in the rice is converted to 1 µg retinol) in U.S. adults. A single 100 g (uncooked) portion of Golden Rice can provide about 500-800 µg retinol, which fulfills 55-70% of the Recommended Dietary Allowance (RDA) for men and women. Queensland University of Technology has developed transgenic banana cultivars with enhanced provitamin A carotenoid content by expressing the Asupina banana *PSY* (the gene encoding phytoene synthase) gene in Cavendish and cooking bananas (95, 96). Moreover, the Africa Biofortified Sorghum (ABS) initiative was founded to produce transgenic sorghum with a final trait stack for enhanced provitamin A concentration, increased iron and zinc bioavailability, and improved protein digestibility (97).

Sorghum in Africa

Sorghum (bicolor L. Moench) is an ancient grain that is also known as guinea corn. The oldest cultivation record was traced to as early as 3000 B.C. in Egypt (98). Sorghum is a

member of the grass family graminea and can be categorized using different criteria. Grain sorghum, dual purpose sorghum, fodder sorghum, forage sorghum, and sweet stalk sorghum are classified based on their height, grain productivity, and sugar amount in the stem (99). Globally, sorghum is the dietary staple for more than 500 million people in over 90 countries of the semi-arid tropics (100). Sorghum is an important dietary staple for the population of Africa, providing an estimated 145 kcal and 4.3 g protein per capita per day (101). According to 2013 Food and Agricultural Organization (FAO) data, sorghum is the fourth most important cereal crop worldwide and in Africa in terms of production in tonnes (102). Approximately 300 million of Africa's most food insecure people who live in the semi-arid tropics rely on sorghum as their staple crop (103).

Sorghum has high photosynthetic efficiency, high growth rates, low photorespiration rate, low water loss rate, and unusual leaf structure because it is a C4 plant. These plants use the C4 carbon fixation pathway that involves temporary incorporation of CO_2 into a three-carbon product, 3-phosphoglycerate. Sorghum therefore matures rapidly and may provide more than one harvest per year (104). Structurally, sorghum is very similar to maize that has hard and floury endosperm and large fat-rich germ without true hull or husk (105). Thus it can be processed using similar dry and wet milling technologies as maize (106). Sorghum is often cultivated in drought-prone environments and genetically adapted to hot and dry agricultural ecologies where it is difficult for other food grains to grow. Because of its tolerance to drought, water logging, saline-alkali infertile soils, and high temperature, sorghum is often referred to as the "camel of the plant kingdom" (106).

Many Africa traditional recipes describe the processing of sorghum based food. Sorghum tortillas are prepared by processing sorghum in alkali (calcium hydroxide), steeping

and washing the processed sorghum (nixtamal), and cooking grounded nixtamal on a hot surface to form a tortilla by the nixtamalization process. Couscous is a major sorghum food staple in North and West Africa; it is a steamed, agglomerated food. In Kenya, Uganda and Tanzania, stiff sorghum porridge (ugali) can be made by boiling sorghum slurry. Sorghum ogi, one of the most important weaning foods in Nigeria and parts of Ghana, is made from fermented porridge. In Africa, bread and cookies are often made from a grain flour composite containing wheat, sorghum, and millet. Via hydrothermal processing (flaking, puffing, extrusion, and micronizing), various sorghum snack products with high quality and good taste can be produced (107, 108). Because of the versatile utilization of sorghum as a staple food ingredient and many merits of growing sorghum as a grain, sorghum is of high potential for enhancing Africa regional development and improving food security.

Provitamin A biofortification of sorghum

Despite the many advantages of growing and consuming sorghum for the African people, conventional sorghum lines have nutritional flaws. The limitations include low concentrations of provitamin A carotenoids and tocopherols (vitamin E), poor iron and zinc bioavailability due to anti-nutrients such as phytate and tannins, and low protein digestibility due to the cross-linking of sorghum kafirin proteins through their disulfide bonding (109, 110). The Africa Biofortified Sorghum (ABS) initiative was therefore founded to address these nutritional constraints through genetic engineering (110). This collaborative project was supported by the Bill and Melinda Gates Foundation and the Howard G. Buffet Foundation through the Danforth Center with in-kind and monetary support from DuPont Pioneer.

As one of the products of the ABS initiative, transgenic β-carotene-biofortified sorghum (ABS 203) was produced by Agrobacterium-mediated transformation (111, 112). To improve the biosynthesis of β-carotene, the biofortified sorghum contains transgenes DXS (deoxyxylulose 5-phosphate synthase) from Arabidopsis, PSY-1 (phytoene synthase) from maize, and CRT-I (carotene desaturase) from *Erwinia uredovora* with PMI (phosphomannose isomerase) from *Escherichia coli* as the transformation selection marker. As an objective of the Grand Challenges in Global Health grant, HGGT (homogentisate geranylgeranyl transferase) gene from barley was also inserted and expressed (97), resulting in improved vitamin E synthesis to mitigate β-carotene oxidative degradation during storage. Hemizygous ABS 203 was previously shown to have increased concentrations of provitamin A carotenoids (3.1-10 μg/g dry weight), compared with non-transgenic control sorghum (1.0-1.3 μg/g dry weight) (97).

One of the goals of our study is to accurately identify and quantify the carotenoid profile of ABS 203 sorghum. The differences between the ABS 203 event used in our study and the Hemi203 sorghum used in the study by Lipkie et al. (97) include: 1) our sorghum was homozygous in the T2 generation instead of hemizygous in the T1 generation; and 2) our sorghum was grown and harvested in the field instead of the greenhouse.

Vitamin A Equivalence of Foods

Bioavailability of provitamin A carotenoids

In the digestive system, the fates of ingested provitamin A carotenoids can be classified into 3 categories: 1) absorbed by enterocytes and then cleaved to retinal and reduced to retinol; 2) absorbed by enterocytes and then incorporated directly into

chylomicrons with other fat-soluble nutrients for circulation through the lymphatic system into the bloodstream; 3) excreted as unabsorbed nutrients. Knowledge of the percentage of

provitamin A carotenoids dedicated to each fate is crucial in evaluating the efficacy and quality of provitamin A biofortified crops (73). Bioavailability is a general term for describing the fraction of the provitamin A carotenoid that is accessible for utilization in physiological functions, metabolism, and storage (113). The process of converting

Figure 1. The terms that relate to provitamin A bioavailability. PAC refers to provitamin A carotenoids that include but are not limited to β-carotene, α-carotene, β-cryptoxanthin, and β-zeacarotene.

provitamin A carotenoids to retinol (VA) for utilization and the related terms in bioavailability studies were described as **Figure 1** that is modified from (114). In the context of investigating the bioavailability of provitamin A carotenoids, it is important to define the terms "bioaccessibility", "bioavailability", "bioconversion", and "bioefficacy" (115). Bioaccessibility is defined as the fraction of carotenoids released from the food matrix and accessible for absorption. Simulated digestion and dialyzability trials are often used to determine *in vitro* carotenoid bioaccessibility (116). Bioavailability is defined as the fraction of carotenoid that is absorbed and available for utilization. Bioconversion is a specific term for provitamin A carotenoid bioavailability studies and defined as the fraction of absorbed

provitamin A carotenoids that is converted to VA. Bioefficacy combines absorption and bioconversion and is defined as the fraction of ingested dietary provitamin A carotenoids that are absorbed and converted to active retinol. Bioefficacy of β-carotene in a certain matrix is often assessed by measuring the retinyl ester response after a single β-carotene ingestion or estimating total body VA stores after a feeding period in either a human or animal study (117, 118). In humans, total body VA stores can be estimated using the stable isotope (e.g. deuterated retinol) dilution assay technique (119). In animal studies, body VA stores are estimated by directly quantifying liver VA (retinol and retinyl esters) concentration as the liver is the primary site for body VA storage (120, 121).

Typical animal models such as the mouse and rat are not suitable for carotenoid absorption studies because of the high efficiency of cleaving provitamin A carotenoids in the intestine of these species (122-124). Ferrets, preruminant calves, and nonhuman primates have been used as alternative models for carotenoid absorption studies (125-129). However, they have individual limitations and are not widely used in recent years (130, 131). Although none of the animal models completely mimics carotenoid metabolism in humans, gerbils were adapted in recent studies due to the following reasons. Like humans, gerbils absorb β-carotene intact even when ingested in only physiological amounts and then accumulate β-carotene in both serum and tissues (132). Gerbils convert the ingested β-carotene to vitamin A (VA) with similar efficiency to humans (133, 134). Although human dose-response and stable isotope labelling methods are preferred to establish the VA equivalence value for the provitamin A carotenoids in crops, a gerbil model provides greater experimental control and allows the direct quantification of liver VA to determine the true bioefficacy value (130, 135) in a more efficient and less expensive way. In addition, to ensure better-designed clinical

studies, preliminary animal studies were recommended in micronutrient supplementation studies (136).

Factors that affect the bioavailability of carotenoids

The bioavailability of carotenoids within different matrices is highly variable. Bioavailability can be enhanced or inhibited by factors from the host, food components, food processing method, and carotenoid characteristics. de Pee and West (137) reviewed the factors that influence carotenoid bioavailability and summarized them as the mnemonic SLAMENGHI: Species of carotenoids, molecular Linkage, 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, and mathematical Interactions.

Bioavailability of provitamin A carotenoids is crucial for biofortification projects as it reflects the efficiency and potential of the biofortified crop in providing VA to the target population. Food matrix greatly affects the bioavailability of carotenoids. Compared with the carotenoids from chloroplasts in green-leafy vegetables, the carotenoids in endosperm amyloplasts (starch-storing plastids) of grains were more easily released in the human digestive system (138, 139), resulting in a comparatively good VA equivalence value. Generally, food processing and preparation methods can be used to enhance the bioavailability of provitamin A carotenoids because the food matrices are softened and the anti-nutrients are diminished during processing (140). Hotz and Gibson (141) reviewed the effects of thermal processing, mechanical processing, soaking, fermentation, and

germination/malting on enhancing the bioavailability of micronutrients, mainly by softening the food matrices in plant-based diet.

Of the many factors that are mentioned above, dietary fat is one of the most important food components that influences the bioavailability of carotenoids. This is because fat intake is able to stimulate the secretion of bile and pancreatic enzymes for micelle formation. As mentioned above, micelles play an important role in fat-soluble nutrient absorption including carotenoids and VA. Our laboratory observed a greater absorption of carotenoids when salads were consumed with full-fat than with reduced-fat salad dressing (142). No absorption of carotenoids was observed when salads were consumed with fat-free salad dressing in the same study. The type of fat may also affect the absorption efficiency of carotenoids. Compared to a meal rich in sunflower oil, ingestion of β-carotene with a meal rich in beef tallow resulted in a higher appearance of β-carotene in triacylglycerol-rich lipoproteins from postprandial plasma (21).

Vitamin A equivalence of provitamin A carotenoids in foods

The VA equivalence of β-carotene is often used to quantify bioefficacy and typically defined as the amount of ingested β-carotene needed to provide vitamin A activity equivalent to 1 μg of retinol. The low β-carotene bioavailability in conventional plant sources is a major determinant of its VA equivalence. When a non-negligible amount of other provitamin A carotenoids (e.g. α-carotene, β-cryptoxanthin, β-zeacarotene) are detected in the food matrix in a bioefficacy study, they should be mathematically transferred and expressed as β-carotene equivalents to calculate VA equivalence (143).

Two sets of recommended VA equivalence values for β-carotene were established and used. According to the 1967 and 1988 recommendations of FAO/WHO (144), the VA equivalence of β-carotene was estimated to be 3.3:1 for an oil matrix, 6:1 for a mixed diet and 12:1 for other provitamin A carotenoids. The term "retinol activity equivalents (RAE)" was developed by the U.S. Institute of Medicine (IOM) and used to quantify VA equivalence (53). In the context of provitamin A carotenoids derived primarily from mixed vegetable sources in the U.S. diet, RAE values were estimated to be 12:1 by weight for β-carotene and 24:1 by weight for other provitamin A carotenoids. When ingested in low amounts (< 2 mg), 2 µg β-carotene in oil is equivalent to 1 µg retinol (RAE value, 2:1) and 1 µg retinol in oil matrix is equivalent to 1 µg retinol (RAE value, 1:1) in the human body. The lowest VA equivalence value indicates the form of vitamin A that has the highest bioefficacy.

In addition to accurate identification and quantification of the provitamin A carotenoids in β-carotene-biofortified transgenic sorghum (ABS 203), our contribution to the ABS Initiative is to determine the bioefficacy of the β-carotene and the other provitamin A carotenoids in the biofortified sorghum using a gerbil model. An accurate estimation of the VA equivalence for the provitamin A carotenoids in the biofortified sorghum is of importance for the future development of the biofortified crop.

In a gerbil model, we expected a somewhat greater VA equivalence value for β-carotene dissolved in oil than that established by the U.S. Institute of Medicine (2:1 by weight) for human subjects. This is because prior gerbil studies showed greater VA equivalence values for β-carotene dissolved in oil, ranging from 2.4:1 by weight to 4.6:1 by weight (74, 145-147). The above varying values reflect the different experimental designs

(e.g. β-carotene dose amount, animals' age, and the length of treatment period) across the studies.

The VA equivalence value for biofortified maize ranged from 3.2:1 by weight in healthy Zimbabwean men and 6.5:1 by weight in healthy U.S. women to 10.4:1 by weight in Zambian children, many of whom had marginal VA deficiency (86, 87, 148). In gerbil studies of biofortified maize, VA equivalence value ranged from 2.1:1 by weight to 11.8:1 by weight (74, 145, 146, 149). Because both sorghum and maize are members of the grass family, Poaceae, with similar structure (hard and floury endosperm, large fat-rich germ, and no true hulls) (104), it is reasonable to expect similar VA values between them. For Golden Rice that is also a transgenic β-carotene-biofortified grain with a starchy matrix, the VA equivalence values were estimated at 2.3:1 by weight for Chinese children and 3.8:1 by weight for U.S. adults (94, 150). When feeding β-carotene-biofortified cassava to humans, the VA equivalence values of β-carotene were estimated at 2.8:1 (88), 4.5:1, and 4.2:1 (151) in a low-fat, moderate-fat meal and high-fat meal, respectively. In a gerbil study, the VA equivalence of β-carotene was determined as 3.7:1 for β-carotene-biofortified cassava (147). As mentioned above, the provitamin A carotenoids in crops with starchy matrices have VA equivalence values higher than the provitamin A carotenoids in green leafy vegetables. Therefore, we expect a VA equivalence value better (lower) than 12:1 for the provitamin A carotenoids in the ABS 203 β-carotene-biofortified sorghum.

Quantitative Analysis of Vitamin A

VA and its derivatives play a central role in essential physiological functions including normal growth, development, immune function, epithelial integrity, reproduction,

and visual system. The assessment of endogenous VA levels in serum and tissues are crucial to elucidate the mechanisms of digestion, absorption, transportation, storage, excretion, metabolism, and biological functions of both VA and provitamin A carotenoids. The concentration of serum retinol is homeostatically controlled and, therefore, inaccurate to directly determine VA status. However, the measurement of serum retinol in a large population is an important tool for analyzing the VAD prevalence in a region (59). Liver is the primary site for body VA storage, and hepatic VA concentration is considered as the "gold standard" for determining body VA status (152). Accurate quantification of hepatic retinol and retinyl esters is a necessary step in VA assessment in animal models. High-performance liquid chromatography (HPLC) is proven to be an efficient, reliable, and well-established tool for VA separation, identification, and quantification (153, 154).

Previous studies demonstrated the need to be meticulous during the analysis of retinoids. The polyene chain of retinoids is rich in electrons and makes retinoids sensitive to low wavelength light (< 500 nm), trace metals, organic acids, heat, and oxygen. If retinoids are directly exposed to daylight during extraction for a period of time, isomerization occurs (155-157).

There are many published extraction methods, and their details can vary considerably from each other. Careful considerations (158) must be taken to evaluate each step of the procedures, from the selection of the media for liver homogenization to the form in which the sample is to be injected into the HPLC system.

Quantitative analysis of serum vitamin A (retinol)

In most physiological states except the postprandial state, retinol is the primary form of VA in the serum (159). The determination of serum retinol is relatively straightforward. As a classic method, serum samples are mixed with a portion of alcohol for protein precipitation. The internal standard (e.g. retinyl acetate) and antioxidant butylated hydroxytoluene are also added to ensure the quality of the analytical work. Hexane is often added multiple times as the extraction solvent. After centrifuging for phase separation, the organic phases of the extraction system are pooled, evaporated, and reconstituted in organic solvents compatible with the HPLC system for the final identification and quantification.

Quantitative analysis of hepatic vitamin A (retinol and retinyl esters)

Hepatic VA extraction can be more complex because of many interfering components in the sample matrix including fat, membrane proteins, enzymes, phospholipids, and other fat-soluble nutrients (158, 160). These factors may prevent hepatic VA from being completely extracted by organic solvents. Appropriate sample pre-treatments can effectively control the errors from extraction procedures.

Procedures before extraction

Similar to serum VA determination, a typical procedure for hepatic retinoid extraction is the homogenization of liver in water-based buffer followed by precipitation of proteins by adding alcohol (161-163). However, several protocols suggested to avoid water from being present in the extraction system (164, 165). A sample lyophilization pre-treatment was therefore conducted. Because retinoids are partially soluble in water, it is possible that a

portion of retinoids is trapped in the aqueous phase during later hexane extraction (166). Homogenization can also be directly conducted in alcohol (167). A proportionally high volume of alcohol is needed to ensure that the liver sample can be evenly homogenized. Saponification (treating samples with alkali and subsequent water washing) before hexane extraction was recommended in some VA analytical protocols to convert all retinyl esters to retinol for further analysis (168, 169). However, this step might be unnecessary and a source of errors (water washing step may remove some water-soluble retinoids) (170, 171). Injecting residual proteins into the HPLC leads to high system back-pressures and column deterioration (158). Therefore, when homogenizing liver in the water-based buffer, alcohol is still added after homogenization as a water miscible organic solvent to precipitate proteins from the liver sample (161, 163). When an adequate amount of alcohol is present in the homogenization media, an extra protein precipitation step is not necessary (172).

Solvent extraction

After homogenization and protein precipitation, a brief vigorous shaking (162) or a long incubation (167) are needed before solvent extraction. However, this step was either omitted (173), short (159) or not specified (172) in many hepatic VA extraction protocols. A classic method of extracting retinoids is to add water-immiscible organic solvent to the system, and hexane was found to be one of the most efficient solvents for the extraction of VA (174). After another vigorous shaking step, the aqueous phase and the organic phase is separated by centrifugation. The extraction procedure is performed multiple times, and the organic phases (supernatant) are pooled, evaporated, and reconstituted in organic solvents for the final identification and quantification.

<u>Reconstitution solvent and HPLC conditions</u>

After evaporating extracting solvents, liver extracts need to be reconstituted in a small volume of suitable solvent before injecting into an HPLC column (175). Ideally, the reconstitution solvent should dissolve all of the analytes and adapt to the HPLC mobile phase system. Although the mobile phases were often similar (a combination of methanol, MTBE, and water) for the VA analytical methods when C_{30} column is used, various reconstitution solvents (MTBE/methanol mixture (176, 177), MTBE (178), methanol/dichloromethane mixture (179, 180) can be found in different protocols. In a C_{18} column system, methanol (159, 167), isopropanol (172), methanol/isopropanol mixture (181), chloroform/methanol mixture (182) were used as reconstitution solvent for VA analysis. For biofortification studies of provitamin A carotenoids, it is necessary to perform an accurate assessment of the VA content in the animal's liver that also accumulates carotenoids (143, 180). The HPLC C_{30} column is therefore used because of its potential to separate retinoids and carotenoids simultaneously (178, 179). Our research group was the first to use the C_{30} column for the analysis of both retinyl esters and carotenoids (87).

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CHAPTER 3

QUANTIFYING THE BIOEFFICACY OF β-CAROTENE-BIOFORTIFIED SORGHUM USING A MONGOLIAN GERBIL MODEL

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Abstract

β-Carotene-biofortified transgenic sorghum is being developed to alleviate vitamin A deficiency (VAD) in semiarid regions of Africa. Our objective was to quantify the bioefficacy of the biofortified sorghum using a Mongolian gerbil model. Gerbils were fed a vitamin A (VA)-free control diet (45% by wt non-transgenic sorghum flour) for 4 wk; 8 animals were then killed at baseline. The remaining animals were randomly assigned to treatments for 6 wk ($n = 11$ per group): 1) control diet dosed daily with cottonseed oil vehicle; 2) biofortified diet (45% by wt transgenic sorghum flour) (99.6 nmol retinol equivalents/d) dosed daily with cottonseed oil vehicle; 3) control diet dosed daily with β-carotene (84.9 nmol retinol equivalents/d); 4) control diet dosed daily with VA (85.1 nmol retinol equivalents/d). Liver VA stores in the baseline (1.27 ± 0.29 µmol), β-carotene-dosed (1.30 ± 0.26 µmol), and biofortified (1.31 ± 0.23 µmol) groups were not different; liver stores in the vehicle-dosed control group were lower (0.92 ± 0.22 µmol) ($P < 0.01$). Liver stores were highest in the VA-dosed group (2.48 ± 0.23 µmol) ($P < 0.0001$). The calculated bioconversion efficiency for the

β-carotene in the biofortified sorghum (4.5 µg β-carotene to 1 µg retinol) was similar to that of the β-carotene dose (3.8 µg to 1 µg retinol). The β-carotene in the biofortified sorghum effectively restored liver VA stores in an animal model.

Introduction

As one of the most widespread micronutrient malnutrition diseases worldwide, VA deficiency (VAD) is a major cause of preventable childhood blindness and also impairs human immune function, especially affecting the poorest populations in low and middle-income countries (1). Based on the World Health Organization (WHO) threshold definition for biochemical VAD (serum retinol below 0.7 µmol/L), most countries in Sub-Saharan Africa and East Asia have over 20% of preschool-age children suffering from VAD (1). Because most populations in these areas heavily rely on plant-based diets, provitamin A biofortification is a promising approach to alleviate widespread VAD (2).

Provitamin A biofortification approaches (3) have been successfully applied in multiple crops such as maize (4), rice (5), cassava (6), and sweet potato (7) by means of transgenic and/or conventional breeding. According to 2013 Food and Agricultural Organization (FAO) data, sorghum is the fourth most important cereal crop worldwide and in Africa (8). Approximately 300 million of Africa's most food insecure people who live in the semi-arid tropics rely on sorghum as their staple crop (9). However, conventional sorghum lines have nutritional flaws (10), including low concentrations of provitamin A carotenoids and tocopherols (vitamin E), poor iron and zinc bioavailability due to anti-nutrients such as phytate and tannins, and low protein digestibility due to the cross-linking of sorghum kafirin proteins through their disulfide bonding (11). The Africa Biofortified Sorghum (ABS)

initiative was therefore founded to address these nutritional constraints through genetic engineering (12).

As one of the products of the ABS initiative, transgenic β-carotene-biofortified sorghum (ABS 203) was produced by Agrobacterium-mediated transformation (11, 13). To improve the biosynthesis of β-carotene, the biofortified sorghum contains transgenes DXS (deoxyxylulose 5-phosphate synthase) from Arabidopsis, PSY-1 (phytoene synthase) from maize, and CRT-I (carotene desaturase) from *Erwinia uredovora* with PMI (phosphomannose isomerase) from *Escherichia coli* as the transformation selection marker. As an objective of the Grand Challenges in Global Health program, HGGT (homogentisate geranylgeranyl transferase) gene from barley was also inserted and expressed (14), resulting in improved vitamin E synthesis to mitigate β-carotene oxidative degradation during storage. Hemizygous ABS 203 was previously shown to have increased concentrations of provitamin A carotenoids compared with the non-transgenic control sorghum (14).

Like humans, gerbils absorb β-carotene intact even when ingested in only physiological amounts and then accumulate β-carotene in both serum and tissues (15). Gerbils convert the ingested β-carotene to vitamin A (VA) with similar efficiency to humans (16, 17). Although human dose-response and stable isotope labeling methods are preferred to establish the VA equivalence value for the provitamin A carotenoids in crops, a gerbil model provides greater experimental control than in human studies and allows the direct quantification of liver VA to determine the true bioefficacy value (18, 19) in a more efficient and less expensive way. Additionally, to ensure better-designed clinical studies, preliminary animal studies were recommended in micronutrient supplementation studies (20).

To predict the nutritional impact of the β-carotene-biofortified sorghum seeds (ABS 203), we experimentally determined the bioefficacy of the β-carotene and the other provitamin A carotenoids in the biofortified sorghum using a gerbil model. The experimental design was modified based on a previous study (21).

Materials and Methods

Animals

Male Mongolian gerbils ($n = 52$), age 40 days, were obtained from Charles River Laboratories (Kingston, NY). The gerbils were individually housed in polycarbonate cages in a temperature- and humidity-controlled room with a 12 h light-12 h dark cycle. They had free access to water and food. Body weights and food intakes were recorded daily. Animal care procedures were approved by the Iowa State University Committee on Animal Care (COAC).

Sorghum and diets

Field transgenic β-carotene-biofortified ABS203 sorghum plants and their non-transgenic control plants were grown at DuPont Pioneer (Johnston, IA). The seeds were decorticated by gentle rotation with golf balls in a poly drum utility mixer (Model 300UT-PL, Cleform Gilson, Marshalltown Co., Marshalltown, IA) in the Iowa State University Center for Crops Utilization Research (CCUR). The pericarps were then separated by winnowing. Briefly, the mixed seeds and pericarps were poured in a vertical stream into a container in front of a small fan. The pericarps were blown horizontally while the seeds continued to fall vertically into the container. This process was repeated 4 more times to

produce fully decorticated seeds. The decorticated seeds were then milled in a burr mill (Model DK-15, Mahlkonig GmbH & Company, Hamburg, Germany). The exterior of the mill was cooled with dry ice to prevent heat transfer to the milled sorghum. After milling, the sorghum flours were passed through an 850 µm pore sieve. The resulting refined sorghum flours were stored at -70 °C. Aliquots of the control and biofortified sorghum flours were shipped to Covance Laboratories (Madison, WI) for the analysis of the macronutrient composition, including soluble and insoluble fiber (**Table 1**). The remaining flours were then shipped overnight on dry ice to Research Diets, Inc. (New Brunswick, NJ) where they were used as the carbohydrate source in preparing the pelleted animal diets based on AIN-93G (22). For both the 4-week VA-depletion and the 6-week treatment periods, the diets contained 45% (by weight) control non-transgenic or biofortified transgenic sorghum flours (**Table 2**).

β-Carotene and VA reference doses

The β-carotene and VA reference doses in cottonseed oil were prepared from commercially available micro-crystalline suspensions in corn oil (β-carotene 30% FS, product code 04 27233 004; DSM Nutritional Products, Parsippany, NJ; Vitamin A Palmitate, 1.7 million IU/g; DSM Nutritional Products). The daily dose of β-carotene or VA with cottonseed oil (a total of 80 µL) was divided into two equal doses that were given approximately 6 hours apart to avoid over-dosing and the resulting reduction in absorption and/or bioconversion efficiency. The daily dose of β-carotene or VA was based on the mean food intake and the corresponding mean provitamin A intake (in nmol β-carotene equivalents) of the gerbils consuming the β-carotene-biofortified transgenic sorghum on the

previous day. The theoretical VA intake from the provitamin A carotenoids was calculated by assuming 100% bioefficacy, i.e., 1 mol of β-carotene provides 2 mol of VA; 1 mol of other provitamin A carotenoids provides 1 mol of VA (21, 23).

Experimental design

There were four dietary treatment groups (**Figure 1**): 45% control non-transgenic sorghum flour diet dosed with cottonseed oil vehicle; 45% β-carotene-biofortified transgenic sorghum flour diet dosed with cottonseed oil vehicle, 45% control non-transgenic sorghum flour diet dosed with β-carotene in cottonseed oil; and 45% control non-transgenic sorghum flour diet dosed with VA in cottonseed oil. For an initial 4-week VA-depletion period, the animals were fed the control non-transgenic sorghum diet. They were acclimated to the study procedures by twice daily dosing with 40 µL of cottonseed oil using a positive displacement pipette. At the end of the 4-week depletion period and after 12 hours of food deprivation, 8 animals were killed to determine baseline serum retinol and liver VA stores. The remaining gerbils were randomly assigned to 4 experimental groups ($n = 11$ per group) of balanced body weight distribution. The animals were then fed their respective diets for 6 weeks. At the end of the 6-week dietary treatment period and after 12 hours of food deprivation, blood samples were collected via cardiac puncture under CO_2 anesthesia. The serum was isolated by centrifugation. The animals were then killed by CO_2 asphyxiation. Livers were collected, rinsed with isotonic saline, blotted, and weighed. Liver and serum samples were stored at -70 °C until analysis.

Carotenoid analysis of sorghum and diet

A cold saponification method was modified based upon previous methods (24, 25) to accurately quantify the carotenoid contents in sorghum flours and animal diets. About 0.1 g biofortified sorghum flour or ground biofortified diet pellet (0.2 g for wild-type sorghum or ground control diet pellet) was transferred into a 50-mL test tube ($n = 4$). Four mL ethanol containing 1 g/L butylated hydroxytoluene (BHT) and 375 ng internal standard (β-apo-8'-carotenal) were added. After vortexing for 3 min, the samples were incubated in an 85 °C water bath for 6 min. The samples were vortexed for 10 seconds after 3 min of incubation. After incubation, the samples were immediately placed on ice to cool to room temperature. Ethanolic potassium hydroxide (800 µL, 30% w/v) was then added. After vortexing for 5 min, 3 mL HPLC grade water were added and the tube was then vortexed for 30 s. A mixture of petroleum ether/diethyl ether (3 mL in total, 2:1 v/v) containing BHT (1 g/L) was added and the tube was vortexed for 1 min. After centrifugation, the upper phase was transferred to a 16×100 mm test tube. The petroleum ether/diethyl ether extraction was then repeated for another two times. The extracts were combined and evaporated to dryness using a speed vacuum evaporator (Model SPD 131 DDA, Thermo Electron, Milford, MA) with a universal vacuum system (UVS 800 DDA, Thermo Electron). Dried samples were reconstituted with 250 µL methyl *tert*-butyl ether (MTBE) followed by 750 µL methanol; 100 µL were injected into the HPLC-PDA system.

VA and carotenoid analysis of liver and serum

Liver and serum samples were extracted without saponification and analyzed by HPLC-PDA for determination of VA and carotenoid concentrations (26). All samples were processed under yellow light. The whole liver samples were first weighted, and then homogenized in 5 volumes (w/v) of ethanol containing 1 g/L BHT. After a 1 h incubation, an aliquot of homogenate (250 µL) was transferred to a second test tube. Internal standard (retinyl acetate, 375 ng) in ethanol containing 1 g/L BHT was added. Hexane (6 mL) containing 1 g/L BHT was then added followed by vortexing × 1 min. After centrifugation, the upper phase was transferred to a third test tube. The hexane extraction was repeated, and the combined extracts were evaporated to dryness using a speed vacuum evaporator. The extracts were then reconstituted with MTBE followed by methanol (1:3 ratio); 100 µL were injected into the HPLC. To extract serum retinol and carotenoids, an aliquot of 250 µL serum was mixed with 500 µL of ethanol containing 150 ng internal standard (retinyl acetate) and 1 g/L BHT. As for liver, the serum samples were extracted two times with hexane (6 mL) containing 1 g/L BHT, dried using a speed vacuum evaporator, and reconstituted with MTBE/methanol before injecting into the HPLC-PDA. Each liver and serum sample was analyzed in duplicate.

HPLC analysis

The HPLC-PDA system included a 717 Plus autosampler with the temperature control set at 5 ℃, two 515 solvent delivery systems, and a 2996 photodiode array detector (Waters Corporation, Milford, MA). The carotenoids and retinoids were separated on a 5 µm C30 Carotenoid Column (4.6 × 250 mm; Waters Corporation). Analytes were separated by

using methanol/water (96.5:3.5 v/v) containing 1% w/v ammonium acetate (solvent A) and methanol/MTBE/water (40:58:2 v/v/v, solvent B) as mobile phases. The following gradient was used: 0-15 min, 0% to 45% Solvent B; 15-35 min, linear gradient to 55% Solvent B; 35-45 min, linear gradient to 85% Solvent B; 45-65 min, linear gradient to 100% Solvent B. The flow rate was 1.0 mL/min. Chromatograms were generated at 286 nm for phytoene, 325 nm for retinol and retinyl esters, 348 nm for phytofluene, and 450 nm for other carotenoids. All solvents were HPLC grade (Fisher Scientific, Fairlawn, NJ). Calibration standards for α-carotene, lutein, phytoene, phytofluene, β-zeacarotene, zeaxanthin, and zeinoxanthin were purchased from CaroteNature (Lupsingen, Switzerland). β-Carotene, β-apo-8'-carotenal, retinol, retinyl acetate, and retinyl palmitate were purchased from Sigma-Aldrich (St. Louis, MO). Retinyl oleate and retinyl stearate were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Cis-β-carotene isomers were quantified using the all-trans-β-carotene calibration curve and retinyl linoleate was quantified using retinyl oleate calibration curve because retinyl linoleate standard was not commercially available. The liver VA concentrations (in retinol equivalents) reflect retinol and retinyl esters.

HPLC-PDA-tandem mass spectrometry (Agilent 1100 HPLC system coupled to an Angilent 1100 Series photodiode array detector and a Bruker 6300 Series MSD ion trap mass spectrometer) with atmospheric pressure chemical ionization (APCI) interface was used to identify two unidentified carotenoids in the biofortified sorghum seeds (27). The column was maintained at 22 °C, and the autosampler for injecting the samples was maintained at 4 °C. The mass spectrometer was operated in positive ion mode with the source voltage set at 4000 V. Nebulizer pressure was set to 15.0 psi with drying gas (nitrogen) flow rate at 5 L/min. Dry temperature and vaporizer temperature were set at 150 °C and 350 °C. The mass

spectra were obtained between *m/z* values of 100 to 700. Mobile phases and HPLC linear gradient were used as previously described (28).

Data analyses

The VA equivalence values for the β-carotene and the other provitamin A carotenoids in the biofortified sorghum were calculated by comparing the mean liver VA stores at the end of the 6-week treatment period for the vitamin A-dosed group and the biofortified sorghum group. The mean increments in the liver VA stores for these treatment groups were first calculated by subtracting the mean liver VA stores for the control sorghum treatment group. VA equivalence values were expressed as β-carotene equivalents in the sorghum to account for the contributions of provitamin A carotenoids other than β-carotene. Thus, the formulas were as:

Vitamin A (nmol) formed from the biofortified sorghum = (VA intake increment as the difference between the VA dose group and control group/liver VA increment as the difference between the VA dose group and control group) × liver VA increment between the biofortified sorghum group and control group

where liver VA (in retinol equivalents) is the total of retinol, retinyl linoleate, retinyl oleate, retinyl palmitate, and retinyl stearate.

VA equivalence value of the provitamin A carotenoids in biofortified sorghum to VA (by wt) = β-carotene equivalents intake increment between biofortified sorghum group and control group (nmol) × 536.8/VA formed from the biofortified sorghum (nmol) × 286.5

where β-carotene equivalents is the total of trans β-carotene, 9-cis β-carotene, 13-cis β-carotene , and (α-carotene + cis β-carotene)/2 (23, 29, 30).

By using similar calculations, the VA equivalence value for the β-carotene reference dose was determined.

Statistical analyses

Daily body weights and weekly food intake during the 6-week treatment period were analyzed by using a repeated-measures ANOVA linear model with study day as covariate followed by Tukey's post hoc test. The fixed effects were treatment group, study day, and group × study day interaction; the random effects were the animals. An autoregressive order 1 AR(1) covariance structure was applied. Mean daily food intake, serum retinol concentrations, liver weights, and liver VA stores were analyzed by using one-way analysis of variance (ANOVA) followed by independent t-tests with Tukey's adjustment when there were significant differences among groups. For the liver storage rate for carotenoid, a mixed effects model was used to perform all pairwise comparisons with a Tukey's adjustment. Carotenoid was included as a fixed effect. Gerbil was included in the model as a random effect to account for correlation between multiple analyzed carotenoids from the same gerbil. Because the liver storage rates of total β-carotene equivalents and total intact phytoene had unequal residual variances, heterogeneous variances of these two variables were accounted and adjusted in the model. A *P* value < 0.05 was considered significant.

Results

Analysis of the sorghum diet and food intake

An HPLC method was developed to resolve the 15 carotenoids (lutein, zeaxanthin, zeinoxanthin, α-carotene, 9-cis β-carotene, 13-cis β-carotene, trans β-carotene, β-

zeacarotenes, two phytoene isomers and three phytofluene isomers) in the biofortified

sorghum. A representative HPLC-PDA chromatogram and carotenoid profile of the

transgenic β-carotene-biofortified sorghum are shown in **Figure 2** and **Table 3**. The internal

standard (β-apo-8'-carotenal) recovery was 94% ± 4% (means ± SD). Total β-carotene

equivalents (means ± SD) were 9796 ± 51 ng/g flour for ABS 203 biofortified sorghum and

761 ± 32 ng/g for non-transgenic control sorghum.

By using HPLC-mass spectrometry, the unidentified carotenoids in the sorghum

extract were identified as a non provitamin A carotenoid zeinoxanthin and two cis isomers of

the provitamin A carotenoid, β-zeacarotene. The identification of zeinoxanthin commercial

standard was confirmed based on the presence of an intense protonated molecular ion signal

at m/z 553.5 $[M + H]^+$ (**Figure 3**). The identifications of cis β-zeacarotene isomers were

based on both their retention times and their protonated molecular ions at m/z 539.5 ($[M +

H]^+$) (**Figure 4**).

Weekly food intakes during the 6-wk treatment phase did not differ among treatment

groups by repeated-measures ANOVA. The mean daily food intakes (± SD) ranged from

5.28 ± 0.31 g (control group) to 5.67 ± 0.37 g (biofortified group), respectively. The mean

daily food intakes were not significantly different among the treatment groups.

Gerbil body and liver weights

There were no significant differences among the four treatment groups in the body

weights of the growing gerbils (**Figure 5**). Liver weights were also not significantly

different among the treatment groups. The mean liver weights (± SD) were 2.18 ± 0.25 g

(baseline group), 2.32 ± 0.28 g (control group), 2.25 ± 0.26 g (biofortified group), 2.04 ± 0.25 g (β-carotene-dosed group), and 2.22 ± 0.23 g (vitamin A-dosed).

Serum retinol and carotenoid concentrations

The mean serum retinol (± SD) in the baseline (1.43 ± 0.11 µmol/L), control (1.35 ± 0.16 µmol/L), biofortified (1.33 ± 0.12 µmol/L), β-carotene-dosed (1.27 ± 0.14 µmol/L), and vitamin A-dosed (1.37 ± 0.11 µmol/L) groups were not different ($P = 0.13$). The internal standard (retinyl acetate) recovery was 99.9% ± 3.6% (means ± SD). Serum retinol concentration is homeostatically controlled and therefore only falls when animals are severely VA deficient with a markedly low hepatic VA store. We do not expect a significant difference in serum retinol concentration because the study was designed to induce only marginal VA deficiency and all gerbils were observed to be in an overall healthy condition with normal physiological functions. Most carotenoids were not detected in the serum of the gerbils in any of the treatment groups; the exception was several colorless carotenoids that were detected in the serum of the animals in the biofortified group (**Table 4**) including phytoene isomer #1 (0.04 ± 0.01 µmol/L), phytoene isomer #2 (0.10 ± 0.05 µmol/L), and phytofluene isomer #3 (0.02 ± 0.004 µmol/L).

Liver VA and carotenoid contents

The mean liver VA stores (± SD) stores in the baseline (1.27 ± 0.29 µmol), β-carotene-dosed (1.30 ± 0.26 µmol), and biofortified (1.31 ± 0.23 µmol) groups were not different. Liver VA stores in the control group were lower (0.92 ± 0.22 µmol) ($P < 0.03$). Liver VA stores were highest in the VA-dosed group (2.48 ± 0.23 µmol) ($P < 0.0001$)

(**Figure 6a**). The same trend was found when liver VA was expressed as VA concentration (**Figure 6b**). The internal standard (retinyl acetate) recovery was 96.3% ± 3.8% (means ± SD). The calculated VA equivalence values for the provitamin A carotenoids were 4.5 µg (biofortified sorghum group) and 3.8 µg (β-carotene dosed group) β-carotene equivalents to 1 µg retinol (**Table 5**).

Liver β-carotene contents in the β-carotene-dosed group (2.06 ± 0.70 nmol/liver) and the biofortified group (2.29 ± 0.77 nmol/liver) were not significantly different. β-Carotene was not detected in the livers of the gerbils in the baseline, vehicle-dosed control, or VA-dosed groups (**Figure 6c**). Other liver carotenoids included two phytoene, three phytofluene, and two cis β-zeacarotene isomers in biofortified group. (**Table 4**). Carotenoid liver storage rate of gerbils in the biofortified group was calculated by dividing liver carotenoid content by total dietary intake of the corresponding carotenoid in treatment period (21) (**Table 6**).

Discussion

In a VA depletion gerbil model, we determined the calculated VA equivalence for the provitamin A carotenoids in the biofortified sorghum as 4.5 µg β-carotene equivalent to 1 µg retinol (4.5:1 by wt), which is lower than the VA equivalence proposed by the U.S. National Academy of Medicine for dietary β-carotene in mixed foods (12:1 by wt) (31). Sorghum is likely to have similar VA equivalence with other starchy biofortified grains because the food matrix is one of the most influential factors that determines carotenoid bioavailability (32). Compared with the carotenoids from chloroplasts in green-leafy vegetables, the carotenoids from endosperm amyloplasts (starch-storing plastids) in grains were more efficiently released in the human digestive system (33, 34), resulting in comparatively low VA equivalence

values. This value for biofortified maize ranged from 3.2:1 by wt in healthy Zimbabwean

men and 6.5:1 by wt in healthy U.S. women to 10.4:1 by wt in Zambian children, many of

whom were marginally vitamin A deficient (28, 35, 36). In gerbil studies of β-carotene-

biofortified maize, VA equivalence values ranged from 2.1:1 by wt to 6.3:1 by wt (21, 37-

39). Considering that both sorghum and maize are members of the grass family Poaceae with

similar structure (hard and floury endosperm, large fat-rich germ, and no true hulls) (40), it is

reasonable to expect similar VA equivalence values between them. For Golden Rice that is

also a transgenic β-carotene-biofortified grain with a starchy matrix, the VA equivalence

values were estimated to be 2.3:1 by wt in healthy Chinese children and 3.8:1 by wt in

healthy US adults (41, 42).

In the current study, 3.8 µg of pure β-carotene dissolved in oil was equivalent to 1 µg

VA (3.8:1 by wt), which was similar to the 4.5 to 1 vitamin A equivalence of the β-carotene

and other provitamin A carotenoids in the biofortified sorghum. This calculated VA

equivalence value for pure β-carotene in oil was greater than that established by the U.S.

National Academy of Medicine for human subjects (2:1 by wt). Two factors may contribute

to this gap. First, compared with humans, gerbils were reported to have greater VA

equivalence values for β-carotene dissolved in oil, ranging from 2.4:1 by wt to 4.6:1 by wt

(21, 37, 38, 43). Second, the gerbils in the BC dosed group were fed more β-carotene in our

study (approximately 1.76 µmol β-carotene equivalents over the 6-wk treatment period) than

in the above-cited studies (ranging from approximately 0.37 µmol β-carotene equivalents to

approximately 1.48 µmol β-carotene equivalents for the treatment period), resulting in

comparatively higher VA stores. Because the absorption and conversion of provitamin A

carotenoids are under negative feedback regulation through the expression of SCARB1 and

BCO1 genes that respectively encode scavenger receptor type B class I and β-carotene 15-15'-oxygenase, the bioconversion efficiency of β-carotene is inversely related to VA status (44). A relatively higher VA equivalence value for the β-carotene dose in our study was therefore expected.

Assuming that the moisture content of the sorghum seeds was 6.6%, and the cooking retention rates for provitamin A carotenoids were 77% and dry matter content was 22.9% as reported for traditional African sorghum porridge (14, 45), a 200 g serving of sorghum porridge would contain 49 g biofortified sorghum flour and 370 µg β-carotene equivalents. If our vitamin A equivalence value measured in gerbils may be extrapolated to humans, one serving of biofortified sorghum porridge would be expected to provide 82 µg retinol activity equivalents (RAEs) and 39% of the estimated average requirement (EAR) for VA (210 µg RAE/d) for children ages 1-3 years (31).

Accurate identification and quantification of the provitamin A carotenoids in the biofortified sorghum is an essential step in an *in vivo* bioefficacy study. Even a trivial amount of provitamin A carotenoids in the sorghum diet could accumulate and make an impact on whole-body stores of VA over a 6-wk feeding period. Overlooking or mistakenly quantifying provitamin VA carotenoids would overestimate or underestimate the final VA equivalence value. To make definitive identification of the provitamin A carotenoids in the biofortified sorghum, LC-(APCI)MS was used to obtain the molecular mass and mass spectra of the unidentified carotenoids, which were subsequently identified as zeinoxanthin and two *cis* isomers of β-zeacarotene. Zeinoxanthin and α-cryptoxanthin have the same molecular weight and similar chromophore spectrum (46). However, α-cryptoxanthin has provitamin A activity based on its structure whereas zeinoxanthin is classified as a non-provitamin A

xanthophyll (47). Because the C_{30} YMC Carotenoid Column was known to be able to separate zeinoxanthin and α-cryptoxanthin (48), the identification of zeinoxanthin in our sorghum flour was confirmed through coelution with a commercial zeinoxanthin standard. In turn, the identity of the zeinoxanthin standard was confirmed by LC/MS based on the presence of an intense protonated molecular ion signal at m/z 553 $[M + H]^+$. In contrast, α-cryptoxanthin would show a quasi-molecular ion with low relative abundance and an intense fragment ion of m/z 535 $[M + H - H_2O]^+$. The loss of the hydroxyl group of α-cryptoxanthin in the ionization chamber is relatively facile because it is located in the ε-ring allylic to the double bond (27, 49).

After an initial incubation with ethanol, heat treatment (in an 85 °C water bath) and saponification are reported to be essential steps for the complete extraction and HPLC-PDA chromatography of carotenoids in maize (25). Because our sorghum diet contained 7% fat wt/wt, saponification was used to remove unwanted lipids that might interfere with the chromatographic separation. Published methods recommend that an internal standard (e.g. β-apo-8'-carotenal) be added after saponification to avoid low internal standard recovery (21, 50). However, this practice would lead to an underestimation of the carotenoid contents due to the failure to adjust for the carotenoid losses during the heating step, which could be a primary source of β-carotene degradation (51). To add internal standard at the beginning of the extraction protocol as a "true" internal standard, we made modifications to a published protocol (52) based on several other carotenoid extraction methods (53, 54) and conducted a room temperature saponification after the initial incubation of the milled seed with ethanol at 85°C in a water bath. With these modifications, the internal standard recovery was 94% ±

4% (means ± SD). Gerbil livers were homogenized in 100% ethanol, which improved

extraction efficiency compared with homogenization in an aqueous buffer (data not shown).

After 6 weeks of feeding a VA-free diet, the gerbils fed control sorghum had lower

liver VA stores compared with the gerbils killed at baseline ($P < 0.03$). Gerbils fed

biofortified sorghum had total liver VA stores that were not statistically different from those

of the gerbils killed at baseline or the β-carotene-dosed gerbils. However, the total liver VA

stores of the gerbils fed biofortified sorghum were significantly higher than those of the

gerbils fed control sorghum. As expected, liver VA stores were highest in the VA dosed

group ($P < 0.0001$) because preformed dietary VA has approximately 100% bioavailability

(31). Serum retinol concentrations did not differ among the treatment groups as expected

because as mentioned above it is homeostatically controlled in healthy animals. Therefore,

the biofortified sorghum effectively restored liver VA stores in a VA-depleted animal model.

Theoretical VA intake from the provitamin A carotenoids was calculated by assuming

1) 1 mol of *trans* or *cis* β-carotene provides 2 mol of VA; 2) 1 mol of α-carotene or *cis* β-

zeacarotene provides 1 mol of VA. In previous gerbil feeding studies, 9-*cis* β-carotene and

13-*cis* β-carotene were not different compared with *trans* β-carotene in maintaining VA

status (23). Also, α-carotene was shown to have 50% of the provitamin A activity of β-

carotene (29). There is little available data regarding the provitamin A activity of β-

zeacarotene. In a single study using the rat growth curative assay (30), the provitamin A

value of β-zeacarotene was reported to be roughly one-fourth that of β-carotene. However, to

the best of our knowledge, this study has not been replicated. Therefore, the two β-

zeacarotene isomers in our study were assumed to have 50% of the provitamin A activity of

β-carotene based on their chemical structures (one unsubstituted β-ionone ring and one open ring).

Lipkie et al. (14) evaluated the carotenoid profile and *in vitro* micellarization efficiency of ABS 203 sorghum. The carotenoid profile of our ABS 203 was similar to the carotenoid profile that they reported. The differences between our ABS 203 event and the Hemi203 sorghum used in the study of Lipkie et al. (14) were that our sorghum was 1) homozygous in the T2 generation instead of hemizygous in the T1 generation; and 2) grown and harvested in the field instead of in a greenhouse.

As the precursors of lycopene and other downstream carotenoid products, the colorless carotenoids phytoene and phytofluene are accumulated in carotenoid-rich fruits and vegetables (55, 56). Because transgenic β-carotene-biofortified sorghum contains enhanced level of DXS, PSY-1, and CRT-I enzymes, increased amounts of phytoene and phytofluene were detected in the biofortified sorghum flour and diet. Compared with vegetables which are known to be rich sources of colorless carotenoids, β-carotene-biofortified sorghum contains approximately 3 times higher phytoene concentration than carrots, and approximately 3 times greater phytoene and 1.2 times greater phytofluene content than tomatoes (57). A growing body of evidence suggests that these colorless carotenoids may play preventative roles against chronic diseases such as several cancers and cardiovascular disease (58). β-Carotene-biofortified sorghum may be an excellent vehicle to provide these biofunctional phytochemicals to humans.

We found proportionally more total dietary phytoene ($1.9 \pm 0.6\%$) and phytofluene ($1.7 \pm 0.3\%$) accumulated in the livers of the gerbils in the biofortified group compared with their hepatic accumulation of total dietary β-carotene ($0.12 \pm 0.04\%$) (**TABLE 6**). This

finding is consistent with that of Moran et al. (59) who also found that the gerbil liver accumulates a greater percentage of colorless carotenoids as compared with β-carotene, which might be attributed to their different absorption, bioconversion, and clearance rates *in vivo*. If bioconverted β-carotene equivalents (liver VA storage increment contributed by provitamin A carotenoids) were counted in the calculation, the liver storage rate of dietary provitamin A of carotenoids (9.5 ± 5.5%) was significantly higher than that of either phytoene or phytofluene ($P < 0.001$). The above result suggests that colorless carotenoids might have much lower absorption efficiency than that of the provitamin A carotenoids. Interestingly, cis β-zeacarotene was also detectable in the livers of the gerbils in the biofortified group. Instead of being enzymatically cleaved into VA or other apocarotenoids, more intact *cis* β-zeacarotene (0.83 ± 0.12% of the total dietary cis β-zeacarotene) was accumulated in the gerbils' livers compared with the accumulated β-carotene ($P < 0.0001$). Because *cis* isomers of carotenoids show less tendency to accumulate in animals' organs compared with their all-*trans* counterparts (60), our study suggests that dietary *trans* β-zeacarotene will probably deposit in the gerbil's liver to a greater extent than cis β-zeacarotene and have lower provitamin A activity than 50% of that of β-carotene. This hypothesis agrees with the findings of a previous study (30). To our knowledge, this is the first *in vivo* study to determine the bioavailability of β-zeacarotene from a food matrix. However, isotopic labeling technology is needed to confirm the conversion mechanism and the provitamin A activity of β-zeacarotene.

Proximate composition of the sorghum flours is presented in **Table 3**. No major differences were observed between the non-transgenic control and the transgenic biofortified flours. Compared with the wild-type control, the transgenic sorghum had a slightly higher

insoluble fiber content (+13.2%). However, insoluble fiber differences of 16.7% had no effect on the bioefficacy of β-carotene in previous gerbil studies (39). In the 6 week treatment period, the biofortified flour was well tolerated by the group of gerbils that received as much as 45% β-carotene-biofortified sorghum flour in their daily diets.

Conclusions

In summary, this study is the first *in vivo* study of transgenic β-carotene-biofortified sorghum. By using improved analytical methods, we showed that β-carotene and the other provitamin A carotenoids in the biofortified sorghum: 1) effectively restored liver VA stores in a VA-depleted animal model; 2) had efficacy similar to that of a supplemental β-carotene dose in maintaining liver VA stores. Accurate measurement of the provitamin A value of β-carotene-biofortified sorghum using a gerbil model provides sound evidence of bioefficacy in improving VA status and a preliminary basis for future human feeding studies.

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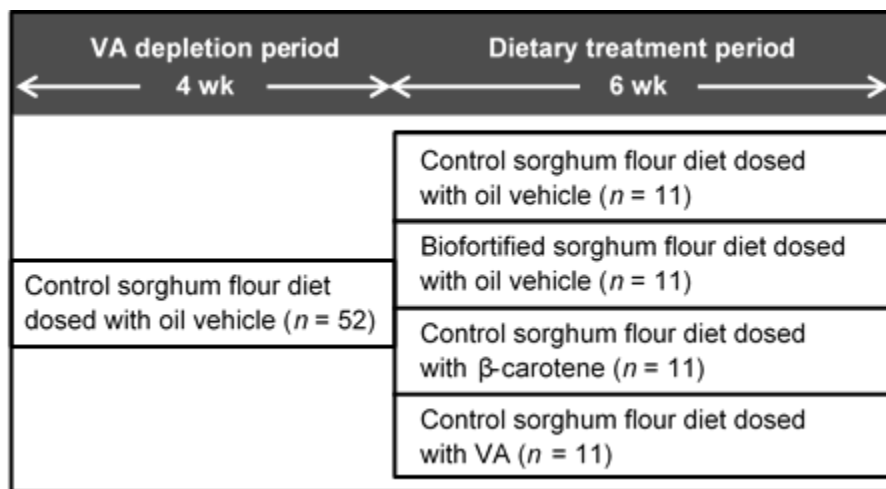
FIGURE 1. Experimental design. The 10-week study was divided into two periods:

1) an initial 4-wk depletion period on a VA-free control sorghum diet; and 2) a 6-wk

dietary treatment period. The daily dose of β-carotene or VA was based on the

mean food intake and the corresponding mean provitamin A intake (in nmol β-

carotene equivalents) of the gerbils consuming the β-carotene-biofortified transgenic

sorghum on the previous day. The gerbils in the baseline group ($n = 8$) were killed

at the end of the 4-wk VA depletion period. The remaining animals were randomly

assigned to 4 treatment groups of balanced body weight distribution ($n = 11$ per

group). These gerbils were killed at the end of the 6-wk dietary treatment period.
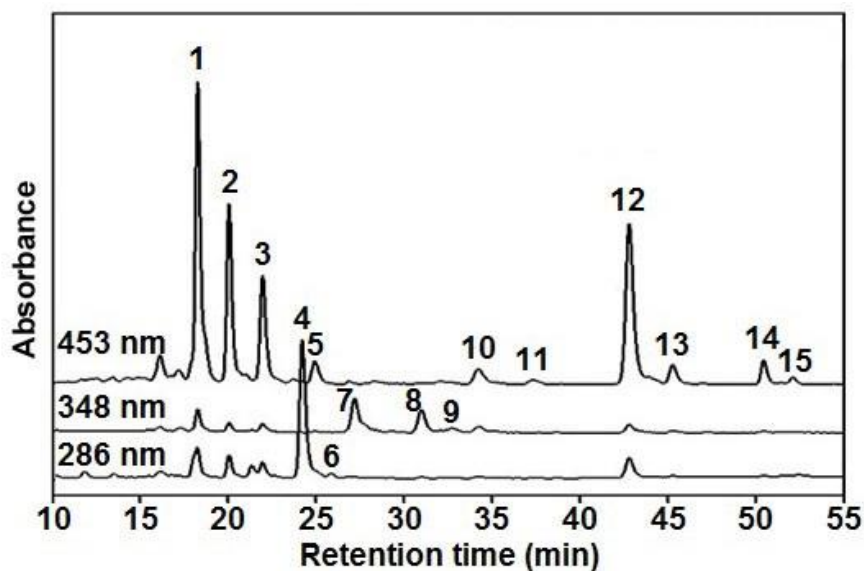
FIGURE 2. HPLC-PDA chromatographic profile of the carotenoids in β-carotene-biofortified sorghum flour detected at 453 nm, 348 nm, and 286 nm. Peaks: 1, lutein; 2, zeaxanthin; 3, β-apo-8'-carotenal (internal standard); 4, phytoene isomer #1; 5, zeinoxanthin, 6, phytoene isomer #2; 7, phytofluene isomer #1; 8, phytofluene isomer #2; 9, phytofluene isomer #3; 10, 13-*cis* β-carotene; 11, α-carotene; 12, *trans* β-carotene; 13, 9-*cis* β-carotene; 14, *cis* β-zeacarotene #1; 15, *cis* β-zeacarotene #2.
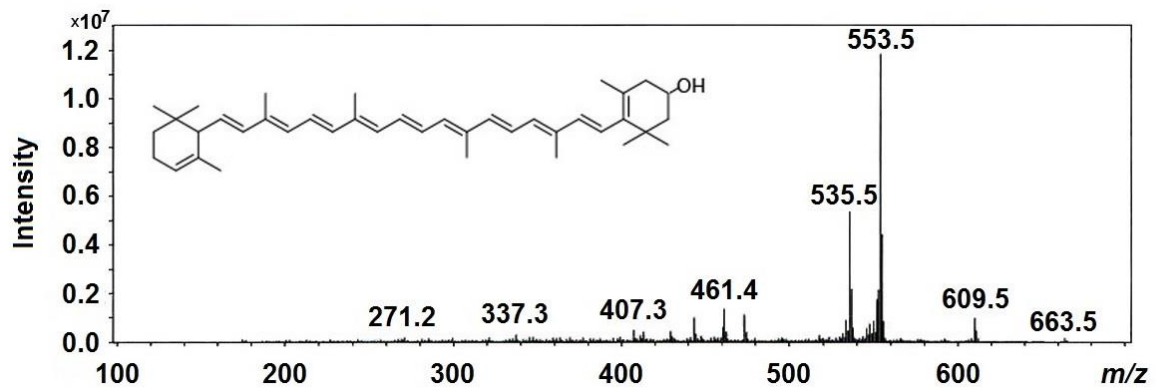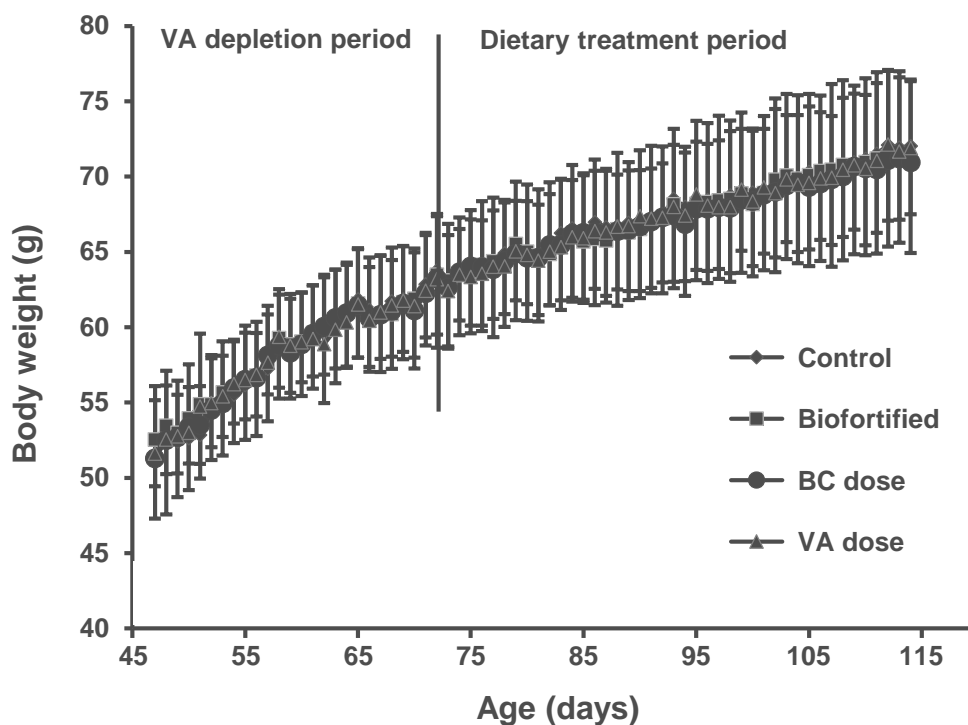
FIGURE 3. Chemical structures and mass spectra of the commercial zeinoxanthin standard.
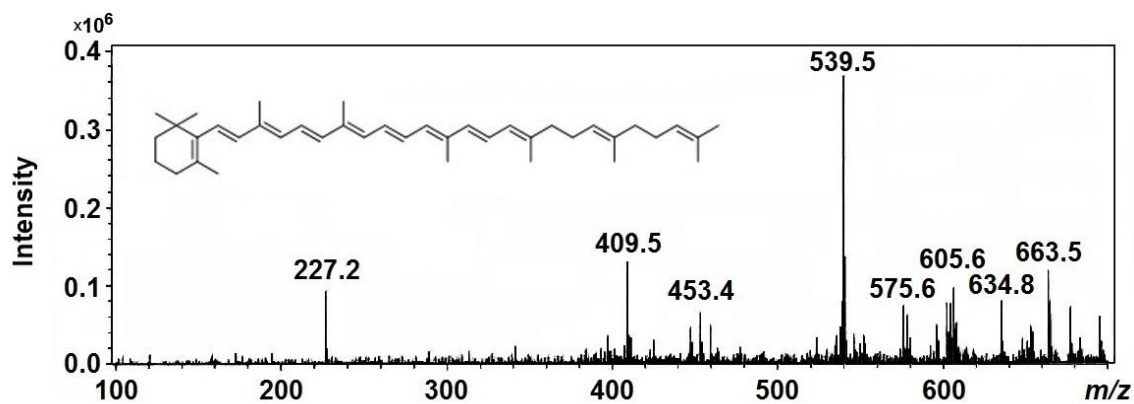
FIGURE 4. Chemical structures and mass spectra of the carotenoid identified as *cis-*β-zeacarotene in the β-carotene-biofortified sorghum.

FIGURE 5. Mean body weights of the gerbils in the treatment groups (n = 11/group): control sorghum diet dosed with oil vehicle (Control); β-carotene-biofortified sorghum diet dosed with oil vehicle (Biofortified); control sorghum diet dosed with β-carotene in oil vehicle (BC dose); and control sorghum diet dosed with VA in oil vehicle (VA dose). No group differences were detected by repeated measures ANOVA with the day as covariate followed by Tukey's adjustment, P < 0.05.
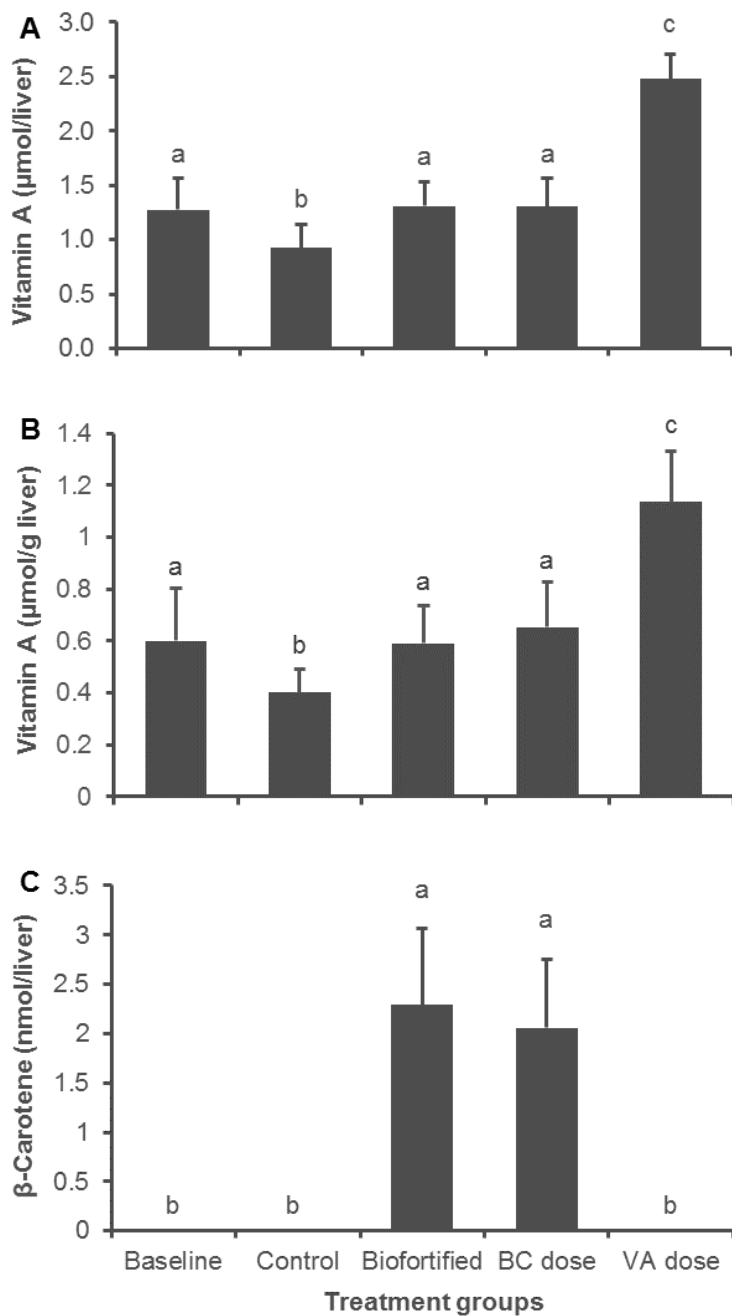
FIGURE 6. Total liver VA (A), liver VA concentration (B), and total liver β-carotene (C) in the: 1) baseline group ($n = 8$) after 4 wk of VA depletion; and 2) treatment groups ($n = 11$/group) after 6 wk of different dietary treatments. Bars having different letters are different by one-way ANOVA, $P < 0.05$.

TABLE 1

Nutritional profiles of the sorghum flours[1]

	Sorghum flour	
	β-Carotene-biofortified	Control
	per 100 g flour	
Calories	367.0	368.5
Calories from fat	34.7	32.4
Fat by acid hydrolysis (g)	3.9	3.6
Carbohydrates (g)	69.6	71.3
Soluble fiber (g)	< 0.75	0.8
Insoluble fiber (g)	11.1	9.8
Total dietary fiber (g)	11.1	10.6
Protein (g)	13.5	12.7
Ash (g)	1.7	1.6
Moisture (g)	11.4	10.8

[1]Analyses were performed by Covance Laboratories, Madison, WI. All values

are means of two replicate measurements.

TABLE 2

Composition of the experimental animal diets [1]

Ingredient	Experimental diet	
	β-Carotene-biofortified	Control
	g/kg diet	
Sorghum flour	450	450
Casein	158	164
L-Cystine	3.0	3.0
Maltodextrin	181	174
Sucrose	110	110
Cellulose	1	3.5
Cottonseed oil	53	53
t-Butylhydroquinone	0.014	0.014
Mineral mix[2]	35	35
Magnesium oxide	1.6	1.6
Vitamin mix (VA-free)[3]	10	10
Choline Bitartrate	2.5	2.5

[1] Provided by Research Diets, Inc. Formulations design was based on AIN-93G (22).

[2] Mineral Mix (22).

[3] Vitamin Mix (AIN-93-VM without VA palmitate) (22).

TABLE 3

Carotenoid concentrations in the sorghum flours[1]

Carotenoid	Sorghum flour	
	β-Carotene-biofortified	Control
	ng/g flour	
Lutein	10393 ± 316	2968 ± 35
Zeaxanthin	5223 ± 126	2433 ± 56
Zeinoxanthin	1049 ± 63	322 ± 21
Phytoene isomer #1	42522 ± 1528	486 ± 30
Phytoene isomer #2	1531 ± 105	ND[2]
Phytofluene isomer #1	2493 ± 49	ND
Phytofluene isomer #2	1589 ± 33	ND
Phytofluene isomer #3	558 ± 25	ND
α-Carotene	298 ± 36	36 ± 4
Cis-β-zeacarotene #1	1304 ± 18	ND
Cis-β-zeacarotene #2	438 ± 27	ND
9-*Cis*-β-carotene	817 ± 19	122 ± 11
13-*Cis*-β-carotene	827 ± 25	104 ± 9
Trans-β-carotene	7132 ± 33	517 ± 18
Total β-carotene	8776 ± 37	743 ± 33
Total β-carotene equivalents[3]	9796 ± 51	761 ± 32

[1] All values are means ± SD; *n* = 4.

[2] ND, not detected.

[3] Calculated assuming that α-carotene and *cis*-β-zeacarotene each have 50% of the

provitamin A activity of β-carotene.

TABLE 4

Phytoene, phytofluene, and β-zeacarotene concentrations in the livers and serum of

the gerbils that consumed the biofortified sorghum[1]

Carotenoid	Liver	Serum
	(nmol/liver)	(nmol/L)
Phytoene isomer #1	71.5 ± 17.8	0.04 ± 0.01
Phytoene isomer #2	86.3 ± 35.6	0.10 ± 0.05
Phytofluene isomer #1	10.9 ± 1.7	ND[2]
Phytofluene isomer #2	5.3 ± 1.0	ND
Phytofluene isomer #3	3.4 ± 0.8	0.02 ± 0.004
Cis β-zeacarotene #1	2.3 ± 0.6	ND
Cis β-zeacarotene #2	0.87 ± 0.19	ND

[1] All values are means $\pm$ SD; $n = 11$.

[2] ND, not detected.

TABLE 5

VA equivalence of the provitamin A carotenoids in the biofortified sorghum[1]

Group	Dietary β-carotene equivalents[2]	Total VA dose	Total β-carotene dose	Theoretical VA intake	Liver VA	VA equivalence BC:retinol
	µg per 6 wk treatment period				*µg/liver*	*by weight*
Control	80 ± 5			85 ± 5	264 ± 63	
Biofortified	1109 ± 74			1183 ± 79	374 ± 65	4.5:1
BC dose	80 ± 8		865	1010 ± 9	373 ± 75	3.8:1
VA dose	83 ± 6	923		1012 ± 7	711 ± 65	

[1] Values are means ± SD; *n* = 11/group.

[2] Calculated assuming that α-carotene and *cis* β-zeacarotene have 50% of the provitamin A activity of β-Carotene.

TABLE 6

Phytoene, phytofluene, and provitamin A carotenoids in the livers and serum of gerbils in biofortified group[1]

Carotenoid	Dietary intake (nmol)	Sample		Liver storage rate (%)[2]
		Liver (nmol)	Serum (nmol/L)	
Total intact phytoene	8490 ± 564	158 ± 51.8	0.14 ± 0.06	1.9 ± 0.6[a]
Total intact phytofluene	1124 ± 75	19.5 ± 3.2	0.02 ± 0.004	1.7 ± 0.3[a]
Total intact β-carotene	1837 ± 122	2.29 ± 0.77	ND[3]	0.12 ± 0.04[b]
Total intact cis β-zeacarotene	386 ± 26	3.19 ± 0.73	ND	0.83 ± 0.19[c]
Total intact α-carotene	70.6 ± 4.7	ND	ND	
Intact β-carotene equivalents	2065 ± 137	3.88 ± 1.11	ND	0.19 ± 0.05[b]
			ND	
			ND	9.5 ± 5.5[d]

[1] All values are means ± SD; $n = 11$.

[2] Mean values within the same column with different superscript letters are different by one-way ANOVA, $P < 0.05$.

[3] ND, not detected.

CHAPTER 4: OPTIMIZING EXTRACTION PROTOCOLS
FOR QUANTIFYING HEPATIC RETINOL AND RETINYL ESTERS

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Abstract

The liver is the primary site for vitamin A storage. Accurate quantification of hepatic retinol and retinyl esters is necessary for the determination of vitamin A status in animal models. Our objective was to optimize methods for the extraction and HPLC analysis of hepatic retinol and retinyl esters. We first compared published non-saponification hepatic vitamin A extraction protocols by using livers collected previously from Sprague-Dawley rats. Based on the results of this comparison, we then investigated the effects of the: 1) homogenization media; 2) duration of vortexing before adding hexane; and 3) reconstitution solvents. Method #1 (J Lipid Res 2014;55:1077-86) resulted in higher hepatic total vitamin A concentrations (423 ± 72.9 nmol/g) than Method #2 (Methods Mol Biol 2010;652:263-75) (36.8 ± 5.35 nmol/g), Method #3 (Anal Methods 2010;2:1320-1332) (347 ± 26.6 nmol/g), and Method #4 (Food Chem 2014;159:477-85) (288 ± 49.1 nmol/g) ($P < 0.0001$). An adequate volume of ethanol added before homogenization of the liver tissue is critical because ethanol is needed in the homogenization media to extract retinoids from liver tissues and then partition them directly to hexane. Homogenizing liver in 100% PBS might facilitate liver tissue binding with water molecules and therefore interfere with the above partitioning process. Adequate duration of vortexing after homogenization and before adding hexane was

shown to be crucial for the exhaustive extraction of hepatic retinol and retinyl esters. The selection of the reconstitution solvent was important for optimal chromatography and therefore for accurate quantification.

Introduction

In mammals, the liver is the major vitamin A (VA) storage site that contains 50% to 80% of the body's total VA (retinol plus retinyl esters) (1, 2). Liver stellate cells contain about 90% of hepatic total VA stores. About 98% of the storage form of VA is present as long-chain fatty acid esters of retinol (3). Serum retinol concentration is homeostatically controlled and therefore not suitable to be used as a precise indicator of body VA stores. Total VA (retinol plus retinyl esters) stores in the liver is considered to be the "gold standard" for determining VA status and is therefore used in animal studies for VA assessment (4). High-performance liquid chromatography (HPLC) is an efficient and reliable tool for VA identification and quantification (5). Numerous bioavailability and physiology studies of provitamin A carotenoids require the accurate assessment of the VA content in the animal's liver that also accumulates carotenoids (6, 7). The HPLC C_{30} column is therefore often used because of its potential to simultaneously separate retinoids and carotenoids (8, 9).

Hepatic VA extraction and HPLC chromatography can be difficult because of interfering components in the sample matrix including fat, membrane proteins, enzymes, phospholipids, and other fat-soluble nutrients (10, 11). These components and the liver tissue structure may prevent hepatic VA from being completely extracted by organic solvents. Appropriate sample pre-treatments can effectively minimize errors due to incomplete extraction. A typical rat liver VA extraction protocol is presented in **Figure 1**.

Saponification before hexane extraction is included in some VA analytical methods to convert retinyl esters to retinol, which simplifies subsequent HPLC analysis by allowing a single measurement of total VA (12, 13). However, some analytical methods exclude the saponification step as unnecessary and a potential source of error due to the degradation of the analytes (14, 15). Thus each step of the analytical procedure should be carefully evaluated from the liver homogenization step to the reconstitution step immediately preceding injection of the sample into the HPLC.

To optimize the hepatic VA extraction method and elucidate the rationale underlying each extraction step, we compared published non-saponification extraction protocols for HPLC analysis of retinol (ROL) and retinyl esters in rodent livers. We then investigated the effects of key factors upon hepatic VA extraction efficiency, including the volume of ethanol added before homogenization of the liver tissue and the duration of vortexing after homogenization and before adding hexane. The selection of the reconstitution solvent was also investigated for optimal chromatography and therefore for accurate quantification.

Materials and Methods

Chemicals and reagents

All solvents were HPLC grade (Fisher Scientific, Fairlawn, NJ). Retinol (ROL), retinyl acetate (RA, internal standard), and retinyl palmitate (RP) were purchased from Sigma-Aldrich (St. Louis, MO). Retinyl oleate (RO) and retinyl stearate (RS) were purchased from Toronto Research Chemicals (Toronto, ON, Canada). Phosphate buffered saline (PBS) was purchased from Life Technologies (Gaithersburg, MD). Unless otherwise mentioned, all other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). HEPES-

KOH buffer contained 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 1 mM ethylenediaminetetraacetic acid disodium (EDTA-Na$_2$) salt solution, 11.5 g/L of potassium chloride (Fisher Scientific, Fairlawn, NJ), and 0.1 mM dithiothreitol. Potassium hydroxide (KOH) was used to titrate the HEPES-KOH buffer to pH 7.4.

Equipment

The HPLC system included a 717 Plus autosampler with the temperature control set at 5ºC, two 515 solvent delivery systems, and a 2996 photodiode array detector (Waters Corporation, Milford, MA). The analytes were separated on a 5 µm C$_{30}$ Carotenoid Column (4.6 × 250 mm; YMC, Allentown, PA). A PowerGen 125 Homogenizer (Fisher Scientific, Fairlawn, NJ) was used for homogenizing the livers (speed 6). A speed vacuum evaporator (Model SPD 131 DDA, Thermo Electron, Milford, MA) with a universal vacuum system (UVS 800 DDA, Thermo Electron) was used to evaporate the combined extracts to dryness.

Animals and diets

Livers from Fischer 344 rats were obtained from Iowa State University Laboratory Animal Resources. Sprague-Dawley (SD) rat livers were generously provided by Dr. Matthew Rowling at Iowa State University. All rodent livers were stored at -80°C and used after thawing completely for 30 min at room temperature.

Method comparison

Livers from 8-9 wk old male Sprague-Dawley rats were used to compare the published methods. Four samples equidistant from the portal vein were taken from each liver

($n = 4$). Four published methods were compared by using each method to analyze one of the four samples from each liver. To isolate the effects of the different extraction protocols and to adapt to the C_{30} HPLC analytical column, the reconstitution solvent used in the different published methods was changed to a unified methyl-tert-butyl ether (MTBE) and methanol (1:3, by vol) mixture. Sample injection volumes for each of the four methods were also standardized for the purpose of comparison.

Method #1 - Homogenizing in ethanol (16). Liver samples (0.1 g) were homogenized in 1 mL ethanol (1.0 g BHT/L) containing 938 ng RA (internal standard) for 15 s. After room-temperature incubation for 1 h followed by vortexing for 5 s, 6 mL hexane (1.0 g BHT/L) was added and vortexed for 3 min. After centrifuging, the hexane extract was transferred to a test tube. Hexane extraction was repeated, and the combined extracts were evaporated to dryness. The extracts were then reconstituted with 625 uL MTBE followed by 1875 uL methanol (1:3). A 100 uL aliquot of the reconstituted extract (1/25 of sample, 0.004 g) was injected into the HPLC system.

Method #2 - Homogenizing in PBS (17). Liver samples (0.1 g) were homogenized in 2 mL PBS for 15 s. A 200 uL aliquot of the homogenate was transferred to a test tube, and 93.8 ng RA (internal standard) in 200 uL ethanol (1.0 g BHT/L) was added. After vortexing for 5 s, 4 mL hexane (1.0 g BHT/L) was added and vortexed twice for 30 s. After centrifuging, the hexane extract was transferred to a second test tube containing 500 uL water. After centrifuging, the hexane layer was transferred to a third test tube and evaporated to dryness. The extracts were then reconstituted with 62.5 uL MTBE followed by 187.5 uL methanol (1:3). A 100 uL aliquot of the reconstituted extract (1/25 of sample, 0.004 g) was injected into the HPLC system.

Method #3 - Homogenizing in PBS/ethanol mixture (18). Liver samples (0.1 g) were homogenized in 2 mL PBS plus an equal volume of ethanol (1.0 g BHT/L) containing 938 ng RA (internal standard) for 15 s. After vortexing for 5 s, 4 mL hexane (1.0 g BHT/L) was added, and the tubes were shaken for 15 min in the dark. After centrifuging, the hexane extract was transferred to a second test tube. Before the next addition of hexane, a brief vortex step was added to disperse the tissue pellet. The hexane extraction was repeated two more times and the combined extracts were evaporated to dryness. The extracts were then reconstituted with 625 uL MTBE followed by 1875 uL methanol (1:3). A 100 uL aliquot of the reconstituted extract (1/25 of sample, 0.004 g) was injected into the HPLC system.

Method #4 - Homogenizing in HEPES-KOH (6). Liver samples (0.2 g) were homogenized in 1 mL HEPES-KOH buffer (50 mM, pH 7.4) for 15 s. A 500 uL aliquot was transferred to a test tube, and 938 ng RA (internal standard) in 500 uL ethanol (1.0 g BHT/L) was added. After vortexing for 5 s, 2 mL hexane (1.0 g BHT/L) was added and vortexed for 3 min. After centrifuging, the hexane extract was transferred to a second test tube. The hexane extraction was repeated two more times and the combined extracts were evaporated to dryness. The extracts were then reconstituted with 625 uL MTBE followed by 1875 uL methanol (1:3). A 100 uL aliquot of the reconstituted extract (1/25 of sample, 0.004 g) was injected into the HPLC system.

Evaluation of individual step of the analytical procedure

Key factors expected to influence liver vitamin A extraction efficiency are summarized in **Figure 1**. Method #3 involves the addition of both ethanol and PBS prior to homogenization. Therefore, we used Method #3 to investigate the effects of changing the

homogenization media. We also investigated the duration of vortexing before adding hexane and the effects of the composition of the reconstitution solvent.

Effects of the homogenization media. Sixteen samples (0.1 g each) were taken equidistant from the portal vein in each of four livers from 29 wk old male F344 rats. The liver samples were then homogenized in either: 1) 4 mL of PBS/ethanol (1:1, by vol); 2) 2 mL of 100% PBS followed by the addition of 2 mL ethanol after homogenization; or 3) 2 mL of 100% ethanol followed by the addition of 2 mL PBS after homogenization. Thus all of these liver samples ultimately contained 1:1 PBS/ethanol before the addition of hexane. PBS and ethanol were chosen for comparison because they are typically used as homogenization media (16-18). The samples were then spiked with 938 ng RA (internal standard) in a small amount of ethanol (approximately 200 µL). To avoid adding extra ethanol to the homogenization media, the internal standard was added after homogenization. These homogenates were then analyzed as specified by the remaining steps of Method #3. For the purposes of comparison, the remaining liver samples were extracted by using Method #1. Method #1 was modified to include three rather than two hexane extractions to promote exhaustive extraction of the hepatic VA.

Effects of the duration of vortexing. One liver from a 29 wk old male F344 rat was used, and six samples equidistant from the portal vein were taken. Liver samples were homogenized in PBS:ethanol (1:1 v/v) as specified by Method #3. A 3 min vortex ($n = 3$) was compared with a 5 s vortex ($n = 3$) to evaluate the impact of the duration of vortexing after homogenization and before adding hexane. Three minutes would typically be considered a relatively long duration of vortexing. The "brief" duration of vortexing

specified in Method #2 was interpreted to be equivalent to about 5 s. These homogenates were then analyzed according to the remaining steps described by Method #3.

Effects of reconstitution solvent. One liver from a 15 wk old male F344 rat was used, and nine samples equidistant from the portal vein were taken. The samples were extracted as described by Method #3 (18). The effects of different reconstitution solvents (total volume 2,000 μL) were then compared: 1) MTBE/methanol (1:3 v/v, MTBE was added and the tube was then vortexed for 5 s before methanol was added) ($n = 3$), 2) 100% methanol ($n = 3$), or 3) 100% isopropanol ($n = 3$). These solvents were chosen for the comparison because they were used for the reconstitution of liver VA extracts in published methods (16, 18, 19).

Linear range for HPLC analysis of liver total VA.

The linear measurement range for HPLC analysis was determined to test if Method #1 could be used for the quantification of livers containing a broad range of VA concentrations. To simulate increasing liver VA concentrations, we used increasing amounts of liver tissue. One liver from a 29 wk old male F344 was used, and 14 liver samples equidistant from the portal vein were taken. Increasing weights of liver tissue (0.03 g, 0.05 g, 0.07 g, 0.09 g, 0.11 g, 0.13 g, and 0.15 g) were extracted in duplicate and their total VA concentration was analyzed by using Method #1. To ensure exhaustive extraction of the VA, Method #1 was modified to include three rather than two hexane extractions. Method #1 was used because it had been found to be the optimal method in the previous method comparison trial.

HPLC analysis

The molar absorbance coefficients (in ethanol) for ROL and RA were 52 770 and 51 180, respectively. A single absorbance coefficient, 49 260, was used for RP, RO, and RS dissolved in ethanol because these retinyl esters have similar structure and absorbance maxima (5, 18, 20). Because a commercial standard was not available, retinyl linoleate (RL) was tentatively identified by comparing its elution order and wavelength of maximum absorbance (λ_{max}) with published references (6, 21). All chromatograms were generated at 325 nm (5, 18). Because the recovery of the internal standard (retinyl acetate) was poor (< 50%) for some methods, external standard curves were prepared from working solutions of commercial standards and used to quantify the corresponding retinoids. The exception was that RL was quantified using the RO calibration curve because a RL standard was not commercially available and because of their similar retention times and chemical structures.

For HPLC analysis, mobile phase A consisted of methanol/water (96.5:3.5 v/v) containing 1 g/L ammonium acetate and mobile phase B consisted of methanol/MTBE/water (40:58:2 v/v/v). The following gradient was used: 0-15 min, 0% to 45% mobile phase B; 15-35 min, linear gradient to 55% mobile phase B; 35-45 min, linear gradient to 85% mobile phase B; 45-65 min, linear gradient to 100% mobile phase B. The flow rate was 1.0 mL/min. Chromatograms were monitored at 325 nm for the analysis of ROL and retinyl esters.

Data analyses

All data were analyzed using SAS software (version 9.4; SAS Institute). Results are presented as means ± SD. The liver total VA concentration (in μg retinol equivalents) reflects retinol and retinyl esters. The recovery of the internal standard was defined as the ratio of the peak area of the injected RA internal standard that had been added to the liver sample to the peak area of the same amount of RA standard directly injected into the HPLC system. The effects of different durations of vortexing before adding hexane were compared by using a Student's unpaired t-test. A simple linear regression model was used to evaluate the relationship between the amount of liver tissue extracted and the quantified liver total VA concentration. A test of lack of fit was performed to determine the adequacy of using this model. Outcomes of interest in the method comparison trial and the other extraction step comparison trials were analyzed by one-way analysis of variance (ANOVA) with Tukey's adjustment followed by independent t-tests when there were significant differences among groups. For the comparison of published methods and the comparison of different homogenization media, a block effect (the livers from different rats) was included as a fixed effect. A P value < 0.05 was considered significant.

Results and Discussion

Method comparison

The hepatic total VA (retinol plus retinyl esters expressed as retinol equivalents) concentrations analyzed by Method # 1 (423 ± 72.9 nmol retinol equivalents/g) were substantially higher than those analyzed by Method # 2 (36.8 ± 5.35 nmol retinol equivalents/g), Method #3 (347 ± 26.6 nmol retinol equivalents/g) and Method # 4 (288 ± 49.1 nmol retinol equivalents/g) (P < 0.0001) (**Figure 2**). The effect of blocking by the

livers from different rats was significant ($P < 0.05$). Similar retinyl ester profiles (liver
concentrations of RP > RS > RO > RL) were detected in the samples that were analyzed by
all four methods (**Table 1**). As expected, the major form of VA identified in the rat livers
was RP for all four methods (22, 23). Method #3 was the most efficient method for
extracting ROL ($P < 0.0001$). Method #2 was more efficient in extracting ROL than retinyl
esters. ROL accounted for 11% of the total VA in the samples extracted by Method #2. In
contrast, only 1-3% of total VA was quantified as ROL for the liver samples extracted by the
other three methods.

Hepatic total VA concentrations analyzed by Method #2 (36.8 ± 5.35 nmol retinol
equivalents/g) were substantially lower than those analyzed by the other three methods
(**Figure 2**). The corresponding recoveries of the retinyl acetate internal standard were also
lowest in the samples analyzed by Method #2 ($47.8 \pm 4.03\%$), compared with those analyzed
by Method #1 ($90.5 \pm 1.03\%$), Method #3 ($81.1 \pm 1.21\%$), and Method #4 ($96.5 \pm 1.89\%$) (P
< 0.0001) (**Table 1**). The low extraction efficiency of Method #2 may be attributable to one
or more of the following key steps within the extraction procedures. First, liver samples
were homogenized in an aqueous medium (PBS), whereas liver samples were homogenized
in ethanol-containing media for Methods #1 and #3. Second, only a "brief" vortex (which
we interpreted as a 5 s vortex) was conducted after adding ethanol, compared with e.g., a 1 h
incubation after adding ethanol in Method #1. Third, unlike the other methods, Method #2
included a water backwash before hexane extraction, which might result in partitioning of the
retinoids between the aqueous and organic phases. Moreover, after addition of hexane, only
a 1 min vortex was applied in Method #2. In comparison, in Method #3, samples were
shaken for 15 min after the addition of hexane. Also, compared with other methods that

contain 2-3 hexane extractions, Method #2 only extracts the sample once with hexane. These factors were then individually investigated to determine their effects on the VA extraction efficiency.

Evaluating extraction protocol steps

Homogenization media. Liver samples are typically homogenized in aqueous solution (PBS or saline), alcohol (ethanol or isopropanol), or a mixture of both before extracting VA into hexane (24). There are few reports that evaluate the effects of the homogenization medium on the efficiency of extracting VA. As shown in **Figure 3**, the difference in VA recovery when comparing different homogenization media was significant ($P < 0.005$). The blocking effect due to the livers from different rats was significant ($P < 0.01$). To isolate the effects of the different homogenization media, ethanol was immediately added after liver samples were homogenized in 100% PBS buffer. PBS buffer was also immediately added after the liver samples were homogenized in 100% ethanol. All of the liver samples therefore ultimately contained 1:1 PBS/ethanol before the addition of hexane. Although the difference was not statistically significant, Method #1, in which livers are homogenized in 100% ethanol, resulted in higher hepatic VA extraction efficiency than the three variations of Method #3. This might be because the modifications of Method #3 each added water during or after liver homogenization, which could interfere with the subsequent transfer of the retinoids to the hexane phase (25). When comparing the three modifications of Method #3, homogenizing liver in 100% PBS resulted in significantly lower extracted VA even when ethanol was immediately added after homogenization (**Figure 3**). Three potential hypotheses might contribute to this phenomenon. First, homogenizing liver in PBS led to

different amorphous liver homogenates (**Figure 4**). The top clear liquid phase was an organic phase (hexane), and the lower light yellow liquid phase was an aqueous phase (ethanol and PBS buffer). After hexane extraction and centrifugation, liver samples homogenized in 100% ethanol with PBS added after homogenization or in a mixture of PBS/ethanol (1:1) became a condensed dehydrated pellet. In contrast, liver samples homogenized in 100% PBS became a liquid layer of fluffy homogenate paste even when ethanol was immediately added after homogenization. This phenomenon suggested that ethanol is needed in the homogenization media to extract retinoids from liver tissues and then partition them directly to hexane. Homogenizing liver in 100% PBS might facilitate liver tissue binding with water molecules and therefore interfere with the above partitioning process. As a result, even if ethanol and hexane were added afterward, the retinyl esters could not be released from the liver tissue easily and extracted by the organic solvents efficiently. Therefore, homogenizing liver in media with adequate ethanol facilitated the retinyl esters and other fat-soluble molecules to be directly taken up by hexane, and a stiff dehydrated pellet was left as the extraction residue.

Second, ethanol increases membrane fluidity by interfering with the packing of molecules in the phospholipid bilayer of the cell membrane (26). Ethanol was therefore shown to be able to disrupt the physical structure of cell membranes. Moreover, the use of solvent mixtures containing alcohol could disrupt the hydrogen bonding and ionic forces between retinoids and associated proteins (27). In addition to the mechanical force provided by the homogenizer, ethanol likely played an additive role by disrupting the biomembranes of liver cells and their organelles, and thereby releasing retinoids that could then be extracted by hexane.

Finally, in the livers of rats that have low vitamin A status (i.e., retinol-depleted rats), retinyl palmitate hydrolase (RPH) accumulates (28). During homogenization, released RPH might catalyze the hydrolysis of retinyl esters and decrease the accuracy of retinyl ester quantification. This enzymatic hydrolysis reaction could be terminated by adding ethanol at a final concentration of at least 50% to denature the endogenous RPH (28). After the liver tissue is homogenized, the possibility of enzymatic degradation of lipids is greatly increased. Therefore, it is recommended that homogenates are immediately inactivated by the addition of alcohol (29). Our results show no difference between homogenization in 50% or 100% ethanol (**Figure 3**). Therefore, homogenizing liver in at least 50% ethanol is recommended. Because ROL is the product of the hydrolysis of retinyl esters, this phenomenon might also explain the higher ROL level that was detected when liver was homogenized in 100% PBS (**Table 2**).

In the homogenization media comparison trial, Method #1 was not superior to Method #3 (**Figure 3**) in extracting hepatic VA. This result was different from what we observed in the method comparison trial (significantly lower VA extraction efficiency for Method #3) (**Figure 2**). This may be because different vortex times were used in Method #3 in the homogenization media comparison trial (3 min vortex before adding hexane) versus the method comparison trial (5 s vortex before adding hexane). The vortex duration was increased in the homogenization media comparison trial in order to isolate the effects of the homogenization media versus the effects of the duration of vortexing. Method #1 has a 1 h ethanol incubation step and it is shown here to be the most efficient method for hepatic VA extraction.

Vortex duration after adding ethanol. Injecting residual proteins into the HPLC system leads to high system back-pressures and column deterioration (10). Therefore, when homogenizing liver in aqueous buffer, ethanol is still added after homogenization as a water miscible organic solvent to precipitate proteins and liberate retinoids from binding proteins (30, 31). As mentioned before, in some protocols, liver was homogenized in media containing ethanol. In both cases, the extent of sample exposure to ethanol, i.e., the vortex duration before adding hexane could be important. However, this vortex step was either omitted (32), brief (17) or of unspecified duration (18) in many VA extraction protocols. Our results indicate that a longer duration of vortexing (3 min vortex) before adding hexane provides significantly higher VA extraction efficiency than a brief duration of vortexing (5 s vortex) before adding hexane ($P < 0.05$) (**Figure 5**). To the best of our knowledge, few published reports have addressed the importance of this step.

Reconstitution solvent. After evaporating the extracting solvent, it is necessary to reconstitute samples in a small volume of suitable solvent before injecting into the HPLC system (33). The reconstitution solvent must be able to dissolve all components of the extract and be compatible with the HPLC mobile phase system. For HPLC methods using a C_{30} analytical column, although the composition of the mobile phases is often similar (a combination of methanol, MTBE, and water), various reconstitution solvents such as MTBE/methanol mixtures (19, 34), MTBE (8), or methanol/dichloromethane mixtures (6, 9) are used with little elucidation. For HPLC methods using a C_{18} column, methanol (16, 17), isopropanol (18), methanol/isopropanol mixtures (35), and chloroform/methanol mixtures (36) were reported as reconstitution solvents for VA analysis. To find an optimal reconstitution solvent for our liver extracts and the C_{30} HPLC column system, we compared

methanol, MTBE/methanol mixture, and isopropanol as reconstitution solvents. As shown in **Figure 6**, 100% methanol provided the best chromatographic resolution for the more polar retinoids, ROL and retinyl acetate (internal standard). However, using 100% methanol as the reconstitution solvent resulted in lower detected total VA concentration ($P < 0.01$) (**Figure 7**), suggesting that pure methanol is a poor solvent for dissolving nonpolar retinyl esters. 100% isopropanol was theoretically miscible with the components of the mobile phase, methanol, MTBE, and water (37), and provided adequate solubility for retinoids (**Figure 7**). However, the resulting chromatographic resolution for the early-eluting peaks (polar retinoids) (**Figure 6**) was below the acceptable standard for our laboratory. MTBE/methanol (1:3 v/v, MTBE was added and the tube was then vortexed for 5 s before methanol was added) was found to provide both acceptable chromatographic resolution for polar retinoids and higher total VA concentrations similar to those measured when reconstituting with isopropanol (**Figure 6, 7**). As a result, the MTBE/methanol mixture (1:3) was chosen as the reconstitution solvent for our trials and is recommended as the optimal reconstitution solvent for VA analysis using the HPLC C_{30} column system.

Other factors that showed no impacts. The water backwash step in Method #2 was considered as a potential factor that might account for the low VA extraction efficiency (29). The brief duration of vortexing during hexane extraction in this method might also influence the partitioning of retinoids from the aqueous phase. However, we did not find significant differences in VA extraction efficiency when we added a water backwash step to Method #1 (400 ± 38.1 nmol/g versus 399 ± 37.6 nmol/g). Similarly, we did not find a significant difference in liver VA concentration when we compared 3 min of vortexing (4711 ± 102 nmol/g) versus the specified 15 min of shaking (4671 ± 26.3 nmol/g) during hexane

extraction in Method #3. Lucas et al. (38) have shown the importance of using hot alcohol

for exhaustive lipid extraction. However, based on Method #1, we did not find a significant

difference between a 5-min hot (boiling) ethanol incubation (192 ± 40.8 nmol/g) and a 1-h

room temperature ethanol incubation (186 ± 49.0 nmol/g). Although repeated hexane

extraction was recommended (10), no difference was found between one time (390 ± 47.1

nmol/g) and two times (400 ± 38.1 nmol/g) hexane extractions in Method #1.

Measurement range for retinyl palmitate and total VA.

Our results indicate that Method #1 was the best method for extracting rat liver VA

because it had the highest extraction efficiency among the four methods (**Figure 2**). The

liver VA content in animals' organs is highly dependent upon their dietary VA content (39).

To determine if Method #1 could be used for VA quantification in livers containing a broad

range of VA contents (e.g., animals of different ages, animals fed diets containing different

levels of VA, and/or animals from different species), the liver from a male F344 rat was used

to determine the linear quantification range of Method #1. For this experiment, to ensure

optimal extraction efficiency, we modified Method #1 to include three hexane extractions

rather than two as specified in the published method. The VA content in this liver was

expected to be high because this rat was of advanced age (29 weeks) and had a high dietary

VA intake (approximately 68 μg retinol activity equivalents per day). Increasing amounts of

liver tissue were extracted (range: 0.03 g to 0.15 g). In this study, regression analysis showed

a nearly perfect linear relationship between the measured liver total VA content and the

weight of liver tissue that was extracted (R-square = 0.99) (**Figure 8**). The slope

corresponding to the linear model was significantly different from zero ($P < 0.0001$). A

linear regression model is adequate because no lack of fit was detected ($P > 0.05$).

Therefore, Method #1 modified to include an additional hexane extraction was found to be

accurate for analyzing rodent livers with widely varying concentrations of VA.

Conclusions

The use of an efficient extraction method when quantifying VA in liver tissue is

crucial in the accurate determination of VA status in animal models. Among four compared

methods, Method #1 was found to have the highest extraction efficiency in terms of total VA.

This method was shown to have a wide range of linearity and thus would be suitable for

analyzing liver samples with varying concentrations of VA. Of the many factors evaluated

within the hepatic VA extraction protocol, an adequate concentration of ethanol (at least

50%) in the homogenization media is necessary for exhaustive extraction. Ethanol is needed

in the homogenization media to extract retinoids from liver tissues and then partition them

directly to hexane. Homogenizing liver in 100% PBS might facilitate liver tissue binding

with water molecules and therefore interfere with the above partitioning process. The initial

extraction of VA from the tissue into ethanol is a critical step that needs to be facilitated by

an adequate duration of vortexing. Finally, the selection of the reconstitution solvent is

important for optimal chromatography and therefore for accurate quantification.

Acknowledgements

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Table 1. Comparison of rat hepatic VA concentrations analyzed using different published extraction protocols[1]

Analyte	Extraction methods			
	Method #1	Method #2	Method #3	Method #4
	nmol/g liver[2]			
Retinol	5.75 ± 0.98^a	4.10 ± 0.66^b	9.48 ± 0.75^c	3.65 ± 0.62^b
Retinyl linoleate	8.83 ± 1.03^a	0.50 ± 0.16^b	6.97 ± 0.54^c	5.66 ± 1.00^d
Retinyl oleate	15.1 ± 2.25^a	0.79 ± 0.08^b	12.4 ± 1.17^c	10.1 ± 1.71^c
Retinyl palmitate	338 ± 57.0^a	26.1 ± 3.71^b	272 ± 17.0^c	227 ± 36.5^c
Retinyl stearate	55.2 ± 13.3^a	5.31 ± 1.30^b	46.1 ± 8.18^{ac}	42.3 ± 10.0^c
Total retinyl esters	417 ± 72.1^a	32.7 ± 5.10^b	338 ± 25.9^c	285 ± 48.7^c
Total VA[3]	423 ± 72.9^a	36.8 ± 5.35^b	347 ± 26.6^c	288 ± 49.1^c
IS recovery (%)[4]	90.5 ± 1.03^a	47.8 ± 4.03^b	81.1 ± 1.21^c	96.5 ± 1.89^d

[1] All values are means $\pm$ SD; $n = 4$.

[2] Mean values within the same row with different superscript letters are significantly different by one-way ANOVA, $P < 0.05$.

[3] The hepatic total VA concentration was equivalent to retinol plus retinyl esters.

[4] Retinyl acetate in ethanol was added at the beginning of the extraction as an internal standard. For Methods #1 and #3, internal standards were added before homogenization as specified in publications. For Methods #2 and #4, internal standard was added after homogenization as specified in the publications.

Table 2. Comparison of rat liver VA concentrations analyzed by methods adding different solvents during liver homogenization[1]

| Analyte	Extraction methods[2]			
	Method #1	Method #3 (P/E)	Method #3 (P)	Method #3 (E)
	nmol/g liver[3]			
Retinol	47.4 ± 6.54^a	60.3 ± 13.4^{ab}	68.0 ± 9.91^b	49.2 ± 9.87^a
Retinyl linoleate	54.9 ± 12.3^a	43.6 ± 12.2^{ab}	33.0 ± 7.14^b	44.24 ± 11.7^{ab}
Retinyl oleate	55.6 ± 10.1^a	45.7 ± 10.9^a	34.2 ± 5.16^b	45.9 ± 8.22^a
Retinyl palmitate	2088 ± 358^a	1727 ± 428^b	1317 ± 211^c	1837 ± 325^{ab}
Retinyl stearate	225 ± 33.3^a	190 ± 42.7^{ab}	153 ± 19.7^b	204 ± 31.7^a
Total retinyl esters	2424 ± 413^a	2005 ± 494^b	1537 ± 242^c	2131 ± 376^{ab}
Total VA[4]	2471 ± 417^a	2066 ± 504^a	1605 ± 244^b	2180 ± 384^a
IS recovery (%)[5]	86.9 ± 1.15^{ab}	89.8 ± 1.71^a	83.9 ± 1.52^b	87.4 ± 2.85^{ab}

[1] All values are means $\pm$ SDs; $n = 4$.

[2] To ensure exhaustive extraction, Method #1: Method #1 was modified to include an additional hexane extraction for a total of three hexane extractions. Method #3 (P/E): Method #3 homogenized liver samples in PBS/ethanol mixture (1:1, vol); Method #3 (P): Method #3 was modified to homogenize liver samples in 100% PBS buffer; Method #3 (E) = Method #3 was modified to homogenize liver samples in 100% ethanol.

[3] Mean values within the same row with different superscript letters are significantly different by one-way ANOVA, $P < 0.05$.

[4] The hepatic total VA concentration was accountable to retinol plus retinyl esters.

[5] For Method #1, retinyl acetate in ethanol was added at the beginning of the extraction as an internal standard. For modified Method #3s, retinyl acetate in ethanol was added after homogenization to exclude the effect of additional ethanol on homogenization medium.
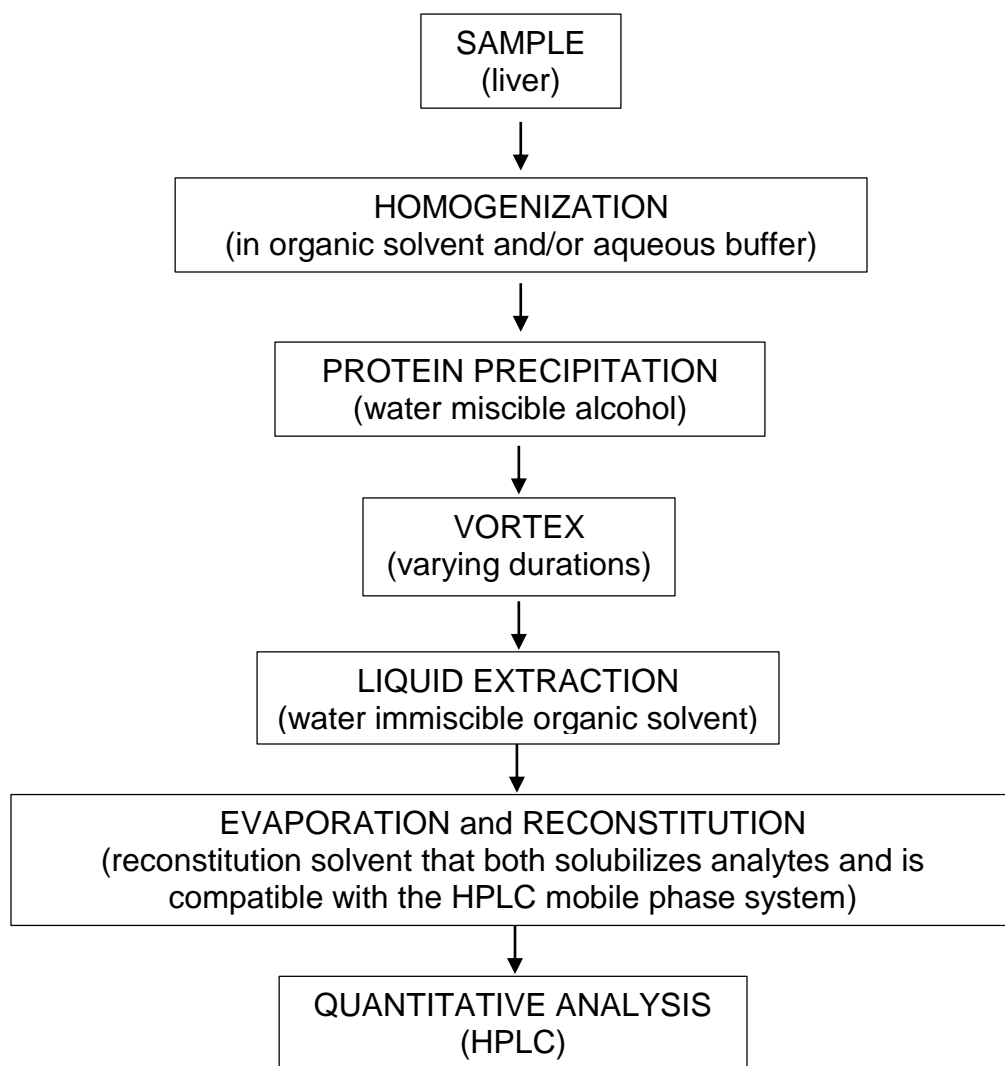
**Figure 1.** Flow chart of the common steps used in liver VA determination. Summarized from (10).
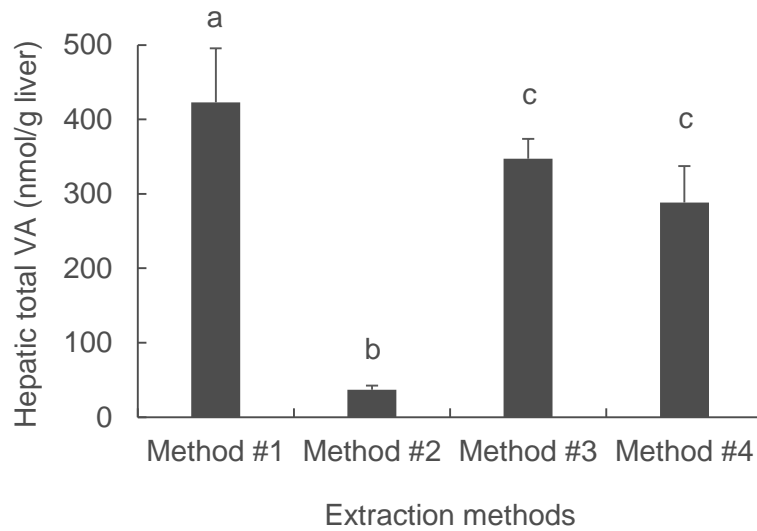
Figure 2. Comparison of rat hepatic total VA (retinol plus retinyl esters) concentrations analyzed using the different published extraction protocols. All values are means ± SD; n = 4. Bars having different letters were different by one-way ANOVA, $P < 0.05$.
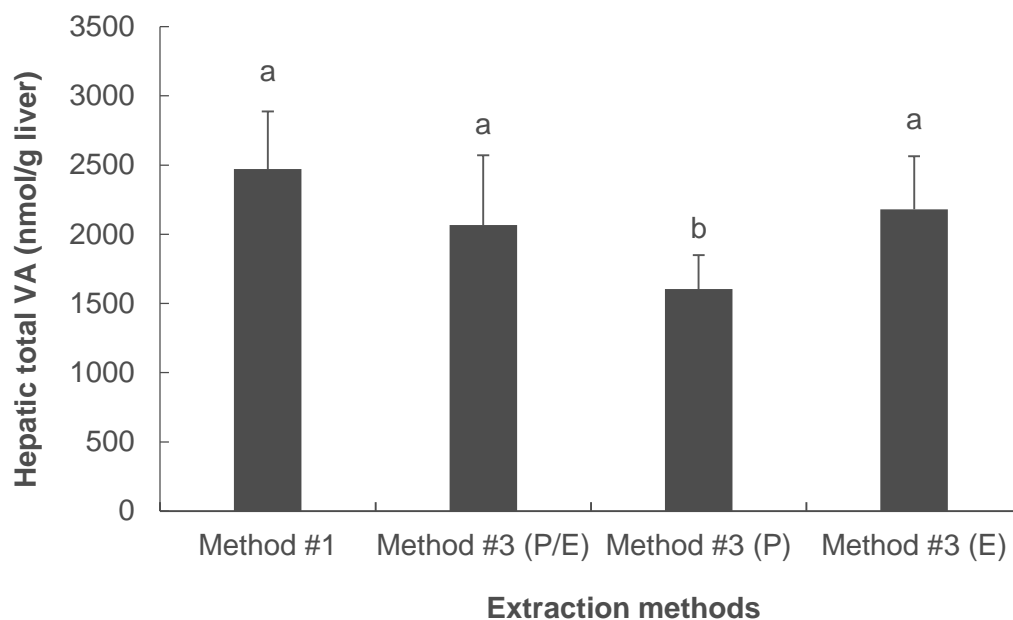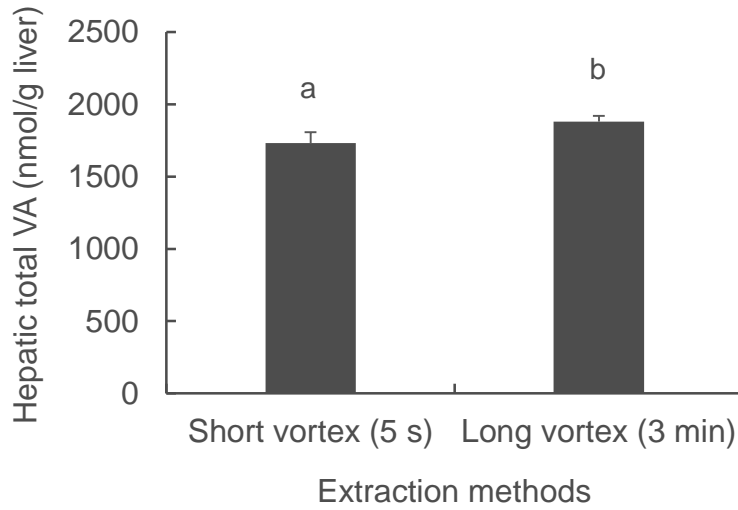
Figure 3. Comparison of rat hepatic total VA (retinol plus retinyl esters) concentrations analyzed by the methods using different homogenization medium. To ensure exhaustive extraction, Method #1 was modified to include an additional hexane extraction for a total of three hexane extractions. Method #3 (P/E): Method #3 homogenized liver samples in PBS/ethanol mixture (1:1, vol); Method #3 (P): Method #3 was modified to homogenize liver samples in 100% PBS buffer; Method #3 (E) = Method #3 was modified to homogenize liver samples in 100% ethanol. All values are means ± SD; n = 4. Bars having different letters were different by one-way ANOVA, P < 0.05.
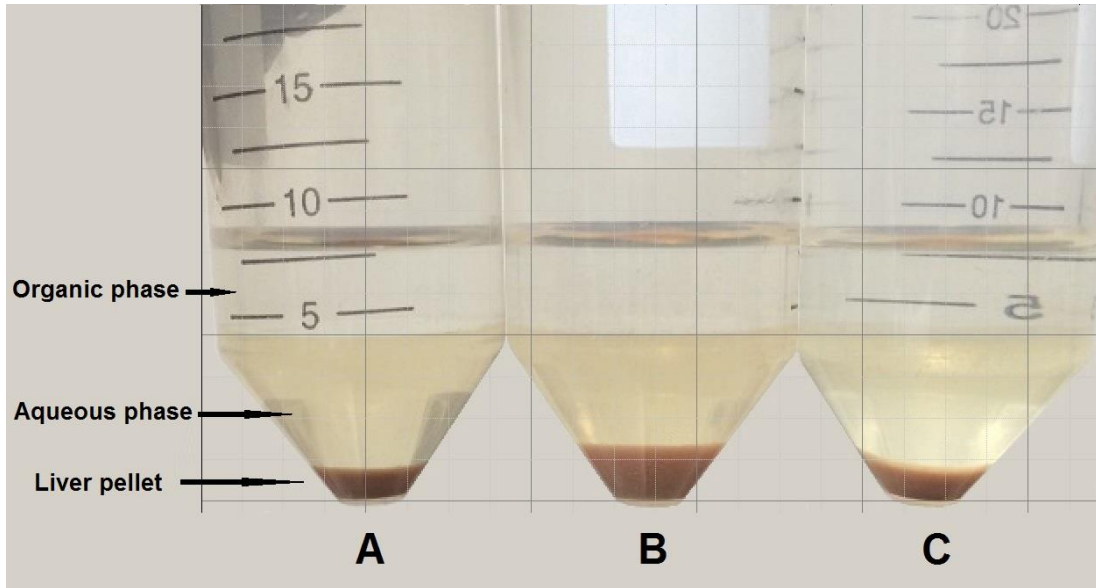
Figure 4. Comparison of rat liver samples homogenized in different homogenization medium. Rat liver samples were homogenized in PBS/ethanol mixture (1:1, vol) (A), 100% PBS buffer (B), 100% ethanol (C). Ethanol was immediately added after the liver samples were homogenized in 100% PBS buffer (B). PBS buffer was immediately added after the liver samples were homogenized in 100% ethanol (C). Hexane (the clear upper layers) was subsequently added to all samples.

Figure 5. Comparison of rat total hepatic VA (retinol plus retinyl esters) concentrations analyzed using methods with different durations of vortexing before adding hexane. A 5 s vortex ($n = 3$) was compared with a 3 min vortex ($n = 3$) to evaluate the impact of the duration of vortexing after homogenization and before adding hexane. All values are means ± SD; $n = 3$. Bars having different letters were different by Student's unpaired t-test, $P < 0.05$.
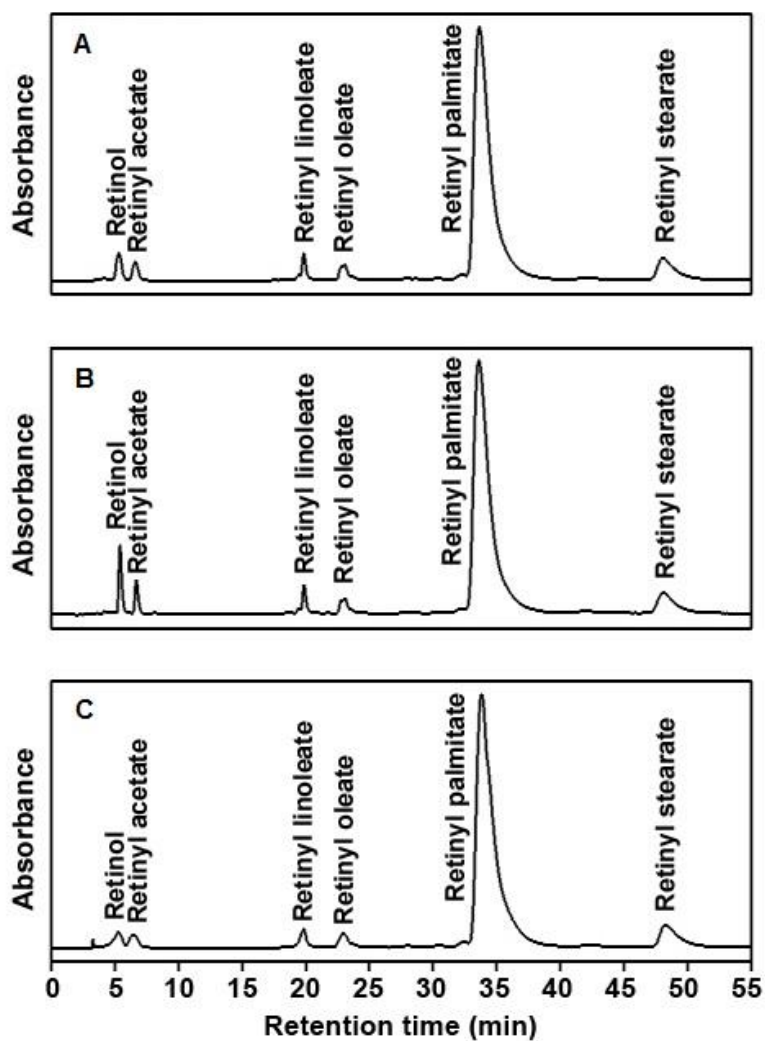
Figure 6. Chromatograms of rat hepatic VA analyzed by different reconstitution methods. MTBE/methanol mixture (1:3 v/v, MTBE was added and the tube was then vortexed for 5 s before methanol was added) (A), 100% methanol (B), and 100% isopropanol (C) were used as reconstitution solvents. HPLC analysis was performed at 325 nm using a C_{30} column.
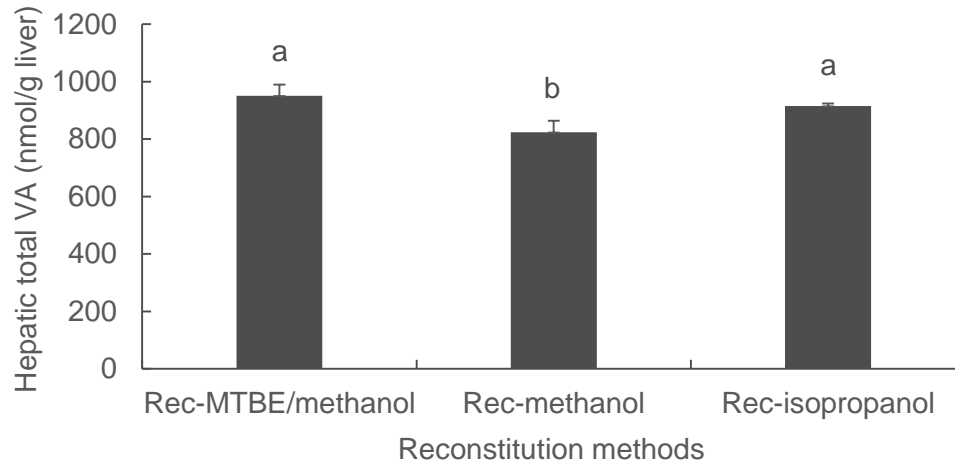
Figure 7. Comparison of rat hepatic total VA (retinol plus retinyl esters) concentrations

analyzed by different reconstitution methods. MTBE/methanol: liver extract reconstituted in

MTBE/methanol (1:3 v/v, MTBE was added and the tube was then vortexed for 5 s before

methanol was added) mixture before injected into HPLC; Methanol: liver extract

reconstituted in 100% methanol before injected into HPLC; Isopropanol: liver extract

reconstituted in 100% isopropanol before injected into HPLC. All values are means ± SD; *n*

= 3. Bars having different letters were different by one-way ANOVA, *P* < 0.05.
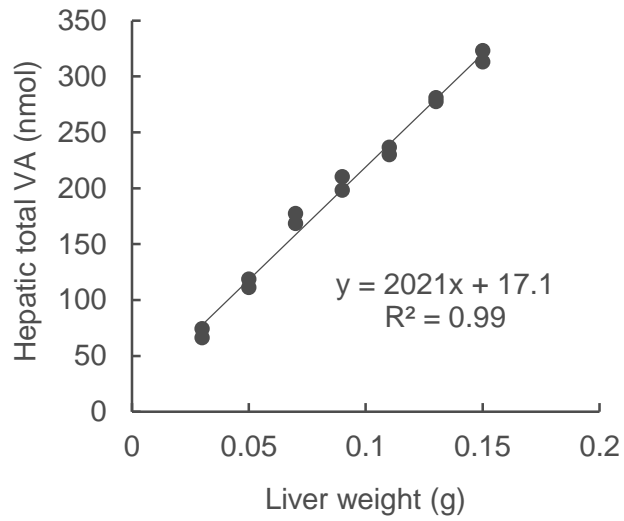
Figure 8. Linear relationship between analyzed hepatic total VA (retinol plus retinyl esters) and extracted liver sample weight.

CHAPTER 5

GENERAL CONCLUSIONS

The studies presented in this dissertation have completed the accurate quantification of the bioefficacy of the provitamin A carotenoids in β-carotene-biofortified sorghum using a Mongolian gerbil model. This study is the first *in vivo* study of transgenic β-carotene-biofortified sorghum. As a result, liver VA stores in the baseline, biofortified, and β-carotene-dosed groups were not different; liver VA stores in the oil vehicle-dosed control group were lower ($P < 0.03$). As expected, liver VA stores were highest in the VA-dosed group ($P < 0.0001$). Serum retinol concentrations did not differ among the treatment groups. The calculated VA equivalence for the provitamin A carotenoids in the biofortified sorghum (4.5 µg β-carotene to 1 µg retinol) was similar to that of the β-carotene dose (3.8 µg to 1 µg retinol). These results demonstrate that β-carotene and other provitamin A carotenoids in the biofortified sorghum 1) effectively restored liver VA stores in a VA-depleted animal model; and 2) had efficacy similar to that of a supplemental β-carotene dose in maintaining liver VA stores.

Assuming that cooking retention rates for provitamin A carotenoids were 77% and dry matter content was 22.9%, as reported for traditional African sorghum porridge (1, 2), a 200 g serving of porridge contains 49 g biofortified sorghum flour and 370 µg β-carotene equivalents. If our vitamin A equivalence value measured in gerbils may be extrapolated to humans, one serving of biofortified sorghum porridge would be expected to provide 82 µg retinol activity equivalents (RAEs) and 27% of the estimated average requirement (EAR) for VA (300 µg RAE/d) for children ages 1-3 years (3).

Accurate identification and quantification of the provitamin A carotenoids in the biofortified sorghum is an essential step in an *in vivo* bioefficacy study. Even a trivial amount of provitamin A carotenoids in the sorghum diet could accumulate and make an impact on whole-body stores of VA over a 6-wk feeding period. Overlooking or mistakenly quantifying provitamin VA carotenoids will overestimate or underestimate the final VA equivalence value. Accurate quantification of hepatic VA concentration is fundamental for the determination of vitamin A status in animal models. There are many published hepatic VA extraction methods, and their details vary considerably from each other. As a result, the selection of hepatic VA extraction method can be very challenging. Several analytical works were therefore conducted to ensure the optimal procedures can be used for our project.

Results from our analytical works can be summarized as follows:

1) In the β-carotene-biofortified sorghum, one zeinoxanthin (non-provitamin A carotenoid) and two cis-isomers of β-zeacarotene (provitamin A carotenoid) could be identified and quantified by LC-(APCI)MS and HPLC-PDA.

2) To add internal standard at the beginning of the extraction protocol as a "true" internal standard, we conducted a room temperature saponification after the initial incubation of the milled seed with ethanol at 85°C in a water bath. Using this modified protocol, the internal standard recovery was 94% $\pm$ 4% (means $\pm$ SD) and the inter-assay coefficient of variation was only 4%.

3) Using a HPLC C_{30} column, a HPLC gradient method was developed to simultaneously separate, identify, and quantify more than 15 retinoids and carotenoids (lutein, zeaxanthin, zeinoxanthin, phytoene, phytofluene, α-carotene, β-carotene, γ-carotene,

β-zeacarotene, retinol, retinyl acetate, retinyl linoleate, retinyl oleate, retinyl palmitate, retinyl stearate).

4)   Method #1 (J Lipid Res 2014;55:1077-86) resulted in higher hepatic total vitamin A concentrations (423 ± 72.9 nmol/g) than Method #2 (Methods Mol Biol 2010;652:263-75) (36.8 ± 5.35 nmol/g), Method #3 (Anal Methods 2010;2:1320-1332) (347 ± 26.6 nmol/g), and Method #4 (Food Chem 2014;159:477-85) (288 ± 49.1 nmol/g) ($P < 0.0001$).

5)   Of the many factors evaluated within the hepatic VA extraction protocol, an adequate concentration of ethanol (at least 50%) in the homogenization media is necessary for exhaustive extraction. Ethanol is needed in the homogenization media to extract retinoids from liver tissues and then partition them directly to hexane. Homogenizing liver in 100% PBS might facilitate liver tissue binding with water molecules and therefore interfere with the above partitioning process. The initial extraction of VA from the tissue into ethanol is a critical step that needs to be facilitated by an adequate duration of vortexing. In addition, the selection of the reconstitution solvent is important for optimal chromatography and therefore for accurate quantification.

In conclusion, by using improved analytical methods, we showed that β-carotene and the other provitamin A carotenoids in the biofortified sorghum: 1) effectively restored liver VA stores in a VA-depleted animal model; 2) had efficacy similar to that of a supplemental β-carotene dose in maintaining liver VA stores. Accurate measurement of hepatic VA and provitamin A value of β-carotene-biofortified sorghum in a gerbil study provide sound evidence of bioefficacy in improving VA status and a preliminary basis for future clinical human feeding studies. The mechanism of ethanol's role in hepatic VA extraction also needs further elucidations.

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