THE EFFECT OF INCORPORATING NUTRIENT-DENSE NATIVE AFRICAN PLANT MATERIALS ON THE BIOACCESSIBILITY OF PROVITAMIN A CAROTENOIDS FROM COMPOSITE CEREAL-BASED FOOD PRODUCTS

by

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A Dissertation

Submitted to the Faculty of Purdue University

In Partial Fulfillment of the Requirements for the degree of

Doctor of Philosophy

Department of Nutrition Science/College of Health and Human Sciences

West Lafayette, Indiana

December 2018

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To my incredible parents:

I can't thank you enough for providing me with enough love to sustain me through all the ups and downs of life.
This work may not compare to all the sacrifices you've made for me, but I dedicate it all to you.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to Dr. Mario Ferruzzi and Dr. Connie Weaver for their constant guidance, support and encouragement. It is truly a privilege to have had the opportunity to work under your guidance. Thank you for patiently pushing me to my fullest potential. Thank you Dr. Ferruzzi for giving me the opportunity to work at the Plant for Human Health Institute and allowing me to expand my research horizon.

I would also like to thank my committee members, Dr. Bruce Hamaker, Dr. Nana Glutsu-Miller and Dr. Juan Andrade for your invaluable insight throughout my graduate career. Thank you for always accommodating me to give me guidance and support.

Current and past fellow lab mates, thank you for your sincere desire to help me and for being my support system throughout the years.

I would like to express my sincere gratitude to my parents for being an incredible example of resilience and commitment. Thank you for making my education your priority. I would not be where I am today if it wasn't for the sacrifices you've made for me. My two brothers, Robera Debelo and Bemnet Debelo, thank you answering my calls and text messages at all hours of the day to help me get through grad school. You are my greatest blessings.

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ABSTRACT

Author: Debelo, Hawi A.. PhD
Institution: Purdue University
Degree Received: December 2018
Title: The Effect Of Incorporating Nutrient-Dense Native African Plant Materials On The Bioaccessibility Of Provitamin A Carotenoids From Composite Cereal Porridges
Committee Chair: Connie Weaver

Vitamin A deficiency is the leading cause of childhood blindness affecting over 190 million preschool children around the world where the highest rates are found in Sub-Saharan Africa (1). The coexistence of this deficiency with shortfalls in iron and zinc has resulted in a shift in intervention strategies from single targeted approach to broader diet diversification. As a result, food-based strategies leveraging local nutrient-dense plants as natural fortificants have gained significant interest for their potential to simultaneously address multiple micronutrient, and in some instances macronutrient, deficiencies. However, the efficacy of such approach depends upon several factors including knowledge on the nutritional composition of native plant materials as well as strategies for their incorporation into staple consumer products. Additionally, there is lack of information on impact of concurrent introduction of mineral and provitamin A rich plants on the stability and bioavailability of each individual nutrients including changes in these factors over extended periods of exposure. This is a key point considering that many of these materials are reported to have potential inhibitors of carotenoid absorption (minerals, fiber and phenolics).

To address these research gaps, this dissertation focuses on three areas including 1) micronutrient, phytochemical and polysaccharide characterization of three commercially available native micronutrient dense African plant materials [*Adansonia digitata* (baobab), *Moringa Oleifera* (moringa) and *Hibiscus Sabdariffa* (hibiscus)] that have been targeted for use as natural iron fortificants; 2) determination of the impact of these materials on the bioaccessibility and intestinal uptake of provitamin A carotenoids from model composite cereal products and 3) assess the effect of longer term exposure to baobab and moringa on provitamin A carotenoid absorption and cellular differentiation biomarkers of human intestinal Caco-2 cells to better understand the potential impacts of extended exposure periods on long term micronutrient uptake.

Characterization of the plant fortificants focused on understanding both nutritive components and potential limiters of carotenoid bioavailability. Baobab, moringa and hibiscus all

were found to contain key phytochemical and polysaccharide components that could be leveraged as nutritional and function ingredients. The relatively higher levels of lutein (57 ± 4.6 μg/g), zeaxanthin (11 ± 0.1 μg/g) and β-carotene (20 ± 2 μg/g) in moringa leaf powder support the notion that this plant material can be used as a source of provitamin A and non-provitamin A carotenoids. Phenolic analysis revealed the presence of substantial amounts of flavan-3-ols (1234 ± 16 mg/100g) in baobab, anthocyanins (2001 ± 56 mg/100g) in hibiscus, and flavonols (5352 ± 139 mg/100g) in moringa leaf powder. Polysaccharide analysis demonstrated that the primary monosaccharide in baobab was found to be xyloglucan (47 %) which is in agreement with the tentative identification Xyloglucans (hemicellulosic polysaccharide) based on linkage analysis. Hibiscus was found to contain similar amounts of xylose (20%) and galactose (27%) supporting the presence of similar proportions of xyloglucans and pectic polysaccharides (type I, type II AG, RG I). The main monosaccharide in moringa was found to be galactose (36%) followed by glucose (23%) and linkage analysis revealed the presence of high proportions of pectic polysaccharides (type I, type II AG, RG I). These results provide insight into presence of potential enhancer or inhibitors of target micronutrient (provitamin A carotenoids or iron/zinc) bioavailability when used as functional and nutritional food ingredients.

Subsequently, the impact of mineral-rich baobab formulated at levels relevant for iron fortification on the bioaccessibility of provitamin A carotenoids (proVAC) from composite millet porridges containing dried carrot and mango was assessed using in vitro digestion. Proportions of millet flour and plant materials were dry blended to deliver ~25% of the RDA for vitamin A(VA) and iron(Fe) as follows: decorticated extruded millet (Senegalese *Souna var.*) (40-60%), dried proVA rich carrot and mango blend (30%), and dried Fe and ascorbic acid rich *Adansonia digitata* (baobab) (0-25%). While there were no significant differences in proVAC bioaccessibility from porridge formulations with 5 and 15% baobab (18.8±2.0 and 18.8±2.0% respectively) as compared to control containing no mineral-rich plant (23.8 ± 1.2%), 25% baobab resulted in a significant decrease ($p<0.05$) in bioaccessibility of proVAC (13.3±1.6%). However, baobab inclusion did not impact intestinal uptake efficiency of provitamin A carotenoids by Caco-2 human intestinal cells (3.3-3.6% α-carotene and 3.7-4.5% for β-carotene) across all formulation. These results suggest that any potential negative effects of baobab inclusion may be limited to food matrix interactions and digestion. This was confirmed in separate experiments that with experiments on baobab and carotenoid blends showing that digested baobab did not affect carotenoid absorption by Caco-2

cells. Overall these data support the notion that that modest inhibition of carotenoid bioaccessibility by baobab may not significantly limit carotenoid delivery from composite porridges. Furthermore, bioaccessible provitamin A content of a serving (200 g) of composite porridges can provide 27 - 48% of the RDA of vitamin A for children 1-3 years of age.

Finally, we evaluated the impact of long-term exposure to baobab and moringa digesta on Caco-2 cell differentiation biomarkers and provitamin A uptake to gain insight into how inclusion of these materials in to a daily diet may alter absorption and transport of nutrients or otherwise have potential negative effects on the intestine. Based on NMR analysis of intracellular metabolites in differentiating Caco-2 monolayers, significant alterations in specific osmotic pressure regulators, particularly glycerophosphocholine, taurine and myo-inositol were observed with repeated exposure to all treatment groups including the control (digested 0.9% saline solution). Changes in these metabolites levels have been linked with specific cellular function including protection against hyperosmotic stress and regulation of paracellular permeability of Caco-2 cells. Evaluation of carotenoid uptake comparing acute and acute on repeated exposure to treatment groups demonstrated that there was an overall significant reduction in carotenoid uptake with repeated exposure across all treatment groups including the control. Despite the reduction in carotenoid uptake, mRNA and protein levels of carotenoid transporters (CD-36, SR-B1 and FABP1) were not significantly altered with exposure through differentiation (except for SR-B1 protein levels). Decrease in SR-B1 levels may be due to bile acid accumulation from the digesta matrix which is known to regulate its own biosynthesis by a mechanism that involves the down-regulation SR-B1 expression to protect cells from cytotoxicity. Our results provide some insight into the impact of simulated gastrointestinal fluids alone on provitamin A uptake in this model system which are usually not taken into consideration in most Caco-2 cell studies. However, overall, these findings indicate that the introduction of baobab and moringa at levels relevant for delivery of meaningful levels of iron (15-23% RDA) should not have negative impacts on human intestinal function or carotenoid uptake over chronic use.

Taken together, our findings indicate that the three native Africa plant materials selected for investigation in these studies can be important sources of key micronutrients (iron, zinc and provitamin A carotenoids) and have potential as natural fortificants with application in staple foods such as cereal porridges. Incorporation of these plant materials, do not appear to negatively affect carotenoid bioavailability although there is a potential for their interaction during micellarization

of carotenoids during normal digestion. While *in vivo* studies evaluating the bioavailability of provitamin A carotenoids from such composite formulations are required, these data support the further exploration of such natural fortification strategies in addressing micronutrient deficiencies in local African communities.

CHAPTER 1. REVIEW OF THE LITERATURE

1.1 Introduction

Vitamin A deficiency is one of the most prevalent public health problem in Sub-Saharan Africa. Preschool children and women of childbearing age are placed especially at a higher risk for vitamin A deficiency due to their higher nutritional requirements as well as socio-cultural and economic burdens (2). The World Health Organization (WHO) estimated that among 190 million preschool children who are vitamin A deficient worldwide, Sub-Saharan Africa accounts for 48% of all reported cases (2). Consequently, considerable efforts have been made in the past few decades to tackle Vitamin A deficiency in these regions. The most common strategies to address vitamin A deficiency are supplement distribution, biofortification, and dietary diversification using local agricultural products.

Vitamin A supplementation is an effective approach to treat vitamin A deficiency; it is cost effective and a relatively quick solution. Unfortunately, it is a short-term solution that requires continued support from outside donors and must be repeated frequently if complementary efforts to improve vitamin A status through dietary improvement are not met. In addition, distribution of supplements might be difficult to implement in countries with poor health and road infrastructure. Biofortification is another effective strategy to improve vitamin A status. Biofortification strategies commonly used for vitamin A deficiency involve the enrichment of staple crops through conventional breeding or genetic modifications. However, the use of biofortified crops are met with legal constraints as some argue that genetically-modified crops present risks to the environment and to consumers (3). In addition to regulatory constraints, the process of developing a biofortified crop can take several years before it can be made available to the public.

Dietary diversification is also another effective strategy that incorporates the addition of local agricultural products rich in preformed vitamin A or provitamin A carotenoids into the diet. This approach is often described as a sustainable approach to prevent vitamin A deficiency because it allows individuals to take advantage of local resources which in turn contributes to the economic growth of the region. Preformed vitamin A is found in animal products whereas provitamin A carotenoids are mostly found in plant materials (4). However, animal products are rarely relied upon as a source of vitamin A in developing countries because of their cost. It has been reported that >80% of the dietary intake of vitamin A equivalents in developing countries are from fruits and vegetables. Therefore, there is an urgent need to understand means of optimizing the use of plants sources to meet vitamin A requirements.

The present review provides a brief introduction of vitamin A function, dietary recommendations and toxicity (as part of a published manuscript) as well as carotenoids focused on their classification, absorption and metabolism. The majority of the review will focus on provitamin A carotenoid rich plant sources from Sub-Saharan Africa and their efficacy to meet vitamin A requirements in this region.

1.2 Vitamin A 1,2,3

Vitamin A (VA) is an essential micronutrient that plays an important role in a wide array of physiological processes including the vision, immune response, cell differentiation and proliferation, intercellular communication, and reproduction. By definition, the term vitamin A refers to the fat soluble compound all-*trans* retinol. However, it is commonly used to collectively represent retinol and its active metabolites including retinal, retinyl ester, and retinoic acid. Retinoic acid is one of the more biologically active forms of vitamin A, serving as the natural ligand for the three retinoic acid receptors (RARα, -β, and -γ) and retinoid X receptors (RXRα, -β,

and -γ), regulating the transcription of over 500 genes involved in cell growth and differentiation. The aldehyde derivate of vitamin A, 11-*cis*-retinal, serves as a chromophore in photoreceptor cells of the retina. Photoisomerization of 11-*cis*-retinal to all-*trans* retinal activates a signaling cascade that results in the perception of light in higher animals. Consequently, one of the clinical manifestations of vitamin A deficiency in humans is severe night blindness or xerophthalmia. Retinyl esters, such as retinyl palmitate, do not have documented biological function directly, but serves as storage forms of vitamin A and as substrate for the formation of active metabolites of vitamin A.

Humans and animals must obtain vitamin A from the diet or through supplemental sources. These are primarily derived from animal products that provide preformed vitamin A (retinol or retinyl esters, which the animal has consumed as preformed VA or has converted from provitamin A carotenoids) or from fruits and vegetables as sources of provitamin A carotenoids. Carotenoids, a class of lipophilic hydrocarbons, are secondary plant metabolites responsible for the red, yellow and orange pigments in plants and some microorganisms. While over 600 carotenoids exist in nature, provitamin A activity is limited to those possessing at least one β-ionone ring along with the polyene chain, including β-carotene, α-carotene, and β-cryptoxanthin. Theoretically, metabolism of β-carotene would yield two molecules of retinol, since β-carotene possesses two β-ionone rings, while α-carotene and β-cryptoxanthin would have half the provitamin activity of β-carotene by virtue of their structure containing only one β-ionone ring.

1.2.1 Deficiencies

Vitamin A deficiency (VAD) results primarily from inadequate dietary intake of vitamin A. VAD is one of the most prevalent nutrition-related health problems, affecting an estimated 190 million preschool children worldwide (5). The classical manifestation of VAD is xerophthalmia,

which is a disorder related to dryness of the eye, resulting in a range of ocular expressions of VAD from night blindness, conjunctival and corneal xerosis, to corneal ulceration and keratomalacia, and ultimately blindness if left untreated (5). Other major health consequences of VAD include severely reduced immune competence leading to increased susceptibility to infectious diseases and higher risk of mortality, particularly in children and lactating women (5). Serum retinol, serum retinol binding protein (RBP), and breast-milk retinol have been used to identify populations at risk for VAD. However, there are limitations in using these biomarkers to determine vitamin A status of individuals. Specifically, serum concentration of retinol is homeostatically regulated and remains constant under wide range of vitamin A intake or liver stores. Thus, a significant decrease in serum retinol concentration may not be apparent unless liver reserves of vitamin A are dangerously low. In addition, factors such as acute-phase response to infection and inflammation, pregnancy and other micronutrient deficiencies affect circulating retinol concentrations. While relatively costly and more labor intensive, assessment of liver stores of vitamin A using isotope dilution methodology is considered the most reliable noninvasive measure of vitamin A status. A liver VA store of 0.07 μmol/g has been used as a cutoff for VAD based on an estimated protection from physiological symptoms of VAD (5).

1.2.2 Dietary recommendations

The U.S. National Academy of Sciences Institute of Medicine (IOM) Estimated Average Requirement (EAR) for vitamin A is calculated based on the amount of dietary vitamin A required to maintain adequate liver stores of vitamin A at 0.07 μmol/g (6). The World Health Organization (WHO) and Food and Agriculture Organization of the United Nations (FAO) have proposed values for vitamin A intake based on a composite of indicators of vitamin A deficiency (7,8). Since vitamin A can be supplied by provitamin A carotenoids, the terms *retinol activity equivalents*

(RAE) and *retinol equivalents* (RE) are used by the IOM and FAO, respectively, to equate different sources to retinol, where 1 μg RAE is equivalent to 1 μg retinol, 12 μg β-carotene, or 24 μg α-carotene or β-cryptoxanthin (6), and 1 μg RE is equivalent to 1 μg retinol, 6 μg β-carotene, or 12 μg α-carotene or β-cryptoxanthin (7). The difference in these equivalencies reflect different levels of confidence in updated absorption data. The IOM Recommended Dietary Allowance (RDA) is 300 μg RAE/day for children 1-3 years of age, 750 μg RAE/day for adult females, 900 μg RAE/day for adult men (6). Similarly, FAO/WHO guidelines recommend a daily vitamin A intake of 400 μg RE for 1-3 years of age, 500 μg RE for adult females and 600 μg RE for adult men (8).

1.2.3 Food sources

The richest sources of preformed vitamin A in the U.S. diet include liver, eggs, and fortified dairy and cereal products (roughly 50-3300 μg RAE/100 g). High concentrations of provitamin A carotenoids can be found in yellow/orange vegetables (e.g. carrots, sweet potatoes, pumpkin), yellow/orange non-citrus fruits (e.g. mangos, apricots, papayas), and dark green, leafy vegetables (e.g. kale, spinach, and collards) (roughly 20-900 μg RAE/100 g) (3). In countries where vitamin A deficiency is a public health problem, fortification of staple foods is used for widespread delivery of vitamin A. These foods include sugar, flours from wheat or maize, and vegetable oil, as well as fortified products such as the peanut butter paste intended to combat general severe malnutrition.

1.2.4 Clinical uses

Prophylactic supplementation of vitamin A has been widely used in vitamin A-deficient populations, and current World Health Organization guidelines recommend prophylactic oral administration of vitamin A supplements be given to children of age 6-59 months in regions where vitamin A has been recognized as a public health problem (9). The suggested dose is a single

supplement of 100,000 IU (30 mg RAE) for infants of age 6-11 months, and 200,000 IU (60 mg RAE) vitamin A every 4-6 months for children of age 12-59 months, since VA can be stored in the liver and mobilized as needed. Vitamin A as all-*trans* retinoic acid is used to treat a form of cancer called acute promyelocytic leukemia (APL). The dose is given as 45 mg/m^2/day orally in combination with anthracycline-based chemotherapy and is considered standard of care for APL (10). Vitamin A as retinyl palmitate at doses of 5000 μg RAE/d has been used to slow progression of retinitis pigmentosa, a genetic disorder characterized by breakdown of the retina, though its efficacy in slowing the disease is not universally accepted (11).

1.2.5 Toxicity

Vitamin A toxicity generally occurs after chronic intakes of doses exceeding 10,000-15,000 μg RAE/d, due to the long half-life of vitamin A in the body. Toxicity symptoms include dry skin, headache, anorexia, nausea, bone pain, and cerebral edema. Acute toxicity has also been observed after ingestion of very high doses. The current Tolerable Upper Limit Intake (UL) for preformed vitamin A is 3000 μg/day for adults (6). No UL exists for carotenoids, since high carotenoid intake has not been observed to result in toxicity symptoms, likely due to the relatively lower absorption efficiency of carotenoids as well as regulation of β-carotene conversion to vitamin A by vitamin A status and dose size. The UL levels were established based on reports demonstrating adverse health consequences of chronic consumption of preformed vitamin A leading to teratogenicity and liver abnormalities. Excess preformed vitamin A intake has also been associated with lower bone mineral density leading to increased risk of osteoporosis and bone fracture, however findings remain conflicting and not thoroughly conclusive.

Vitamin A intake in most developed countries often exceeds the RDA or even the UL for most age groups but the levels are usually below the no-observed-adverse-effect level (NOAEL).

In the United States, the 95th percentile of vitamin A consumption from foods and supplements by nonpregnant women of age 19-30 y as well as preschool children exceeds the UL but not the NOAEL of these age groups.

1.2.6 Recent research

Despite the recognition of the prevalence of vitamin A deficiency for decades, the most effective approaches for addressing the problem are still under investigation. Recent research includes studies on methods for biofortification of staple foods to create sustainable strategies for at risk populations, and studies on the effect of these biofortified crops, such as cassava and maize, on vitamin A status of children in various populations. There is also increasing awareness that vulnerable populations may receive multiple forms of supplementation, resulting in hypervitaminosis A, and studies for improving biomarkers to identify this condition are underway. Relating to both vitamin A deficiency and toxicity, there is currently a significant effort underway to better understand equilibration of recently ingested VA with body stores to improve isotope dilution protocols and strategies for assessment of exposure and status after vitamin A interventions (12). Specific to vitamin A function, several studies are investigating the molecular mechanisms behind the potential chemopreventive and chemoprotective properties of vitamin A in different diseases and conditions. Mechanisms of retinoic acid signaling in different morphogenesis is also so being explored. There is also a growing international collaboration to address vitamin A deficiency through generation of new food processing methods to enhance the bioavailability of provitamin A carotenoids from plant sources. Current studies are also investigating methodologies to establish appropriate conversion factors for estimating the bioavailable vitamin A content of plant foods.

1.3 Provitamin A carotenoids

Exclusive of cis-trans isomers, more than 700 carotenoids have been isolated and characterized from natural sources. Carotenoids are a class of hydrocarbons consisting of eight isoprenoid units, joined in a head-to-tail pattern, of two C20 molecules (13). This produces the parent C40 carbon skeleton from which all the individual variations arise. This variation is a result of modification of the skeletal structure by cyclization, hydrogenation, or oxidation. Based on these differences, carotenoids are classified into two groups: hydrocarbons commonly known as carotenes and xanthophylls, the oxygenated derivatives of these hydrocarbons. The alternating double and single bonds in which the electrons are effectively delocalized over the entire length of the polyene chain gives carotenoids a distinctive molecular shape, chemical reactivity, and light-absorbing properties (13). The polyene chain along with at least one β-ionone ring is what determines the vitamin A activity of carotenoids (14). Currently, about 50 naturally occurring carotenoids have been identified as provitamin A molecules. β-carotene, α-carotene, β-cryptoxanthin, lycopene, and lutein are the primary carotenoids found in human plasma among them, β-carotene, α-carotene, and β-cryptoxanthin are provitamin A carotenoids (Figure 1.1) (15). However, as seen in figure 1.1, α-carotene and β-cryptoxanthin have half the activity of β-carotene because they only have one β –ionone (i.e. the other ring structure is modified).

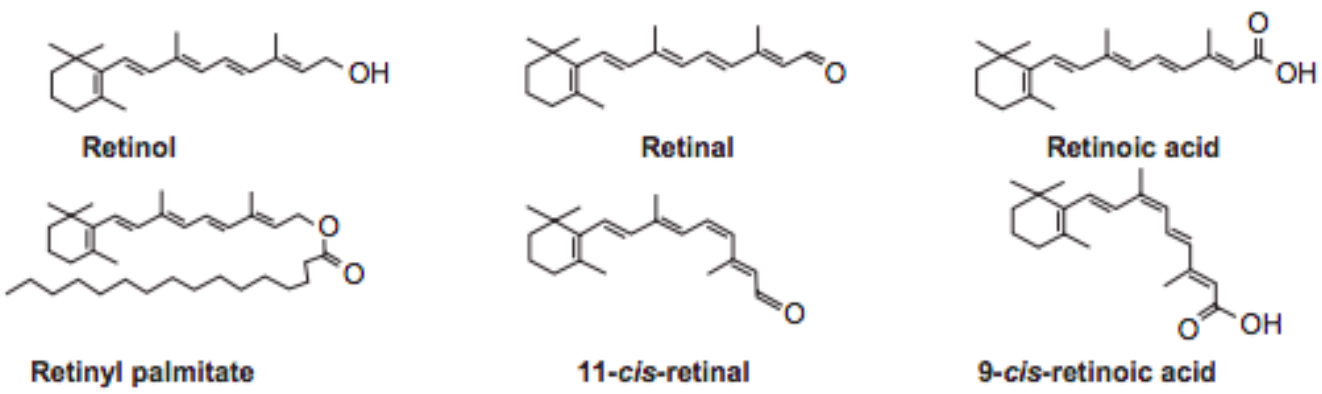

Figure 1.1 Chemical structure of vitamin A (retinol) and its different active metabolites involved in a wide variety of physiological function including vision, immune response, cell differentiation and proliferation, intercellular communication, and reproduction. Adopted from Harrison et al. (2014)

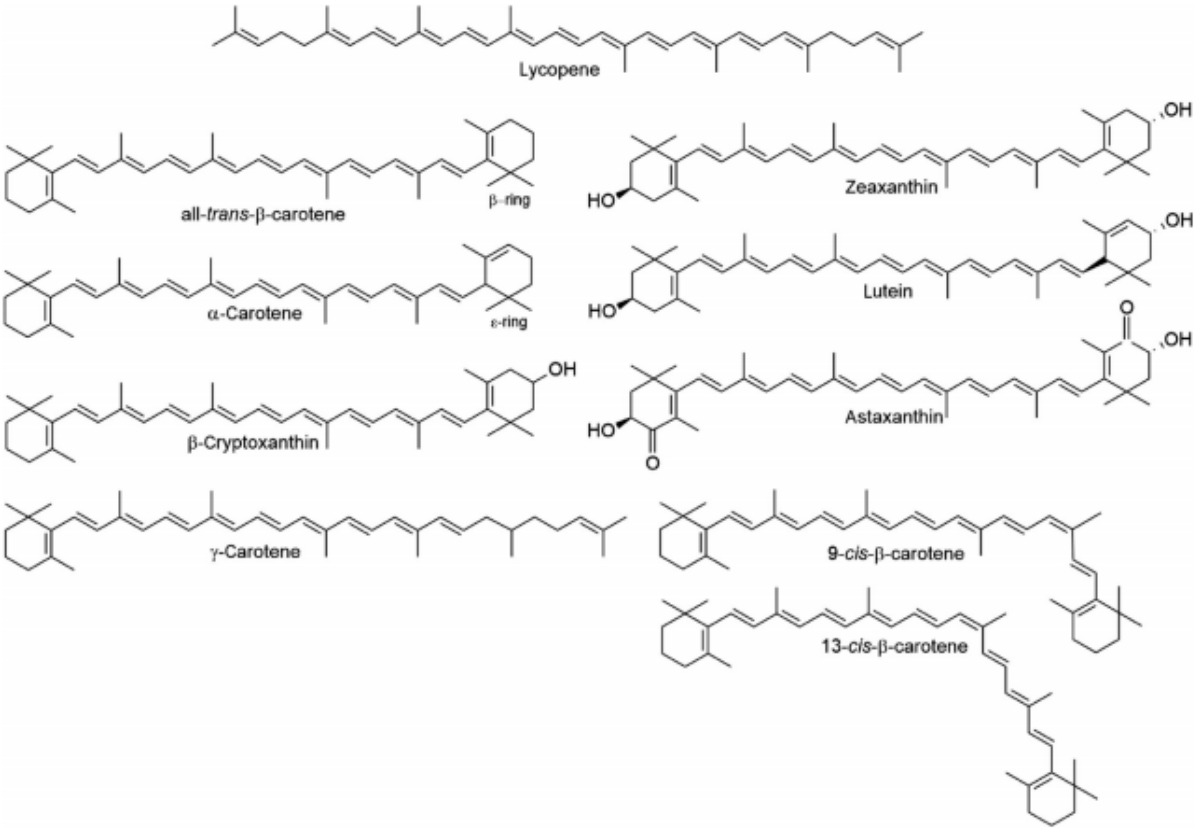

Figure 1.2 Structures of most common dietary carotenoids in the Western diet. Among these carotenoids, β-carotene, α-carotene and β-cryptoxanthin are known to have provitamin A activity due to the presence of beta-ionone ring along with the conjugated double bonds. Adopted from Harrison et al. (2014)

1.3.1 Overview of carotenoid digestion, absorption and metabolism

Once ingested, carotenoids follows the fate of lipids and their absorption occurs in the upper half of the small intestine (**Figure 1.3**) (16). In the initial phase of digestion, carotenoids are emulsified into mixed micelles containing cholesterol, phospholipids, lipid digestion products and bile salts. The micellarization and subsequent absorption of carotenoids has been shown to be higher for polar carotenoids such as lutein as compared to β-carotene (17). This phenomenon is believed to be due to the ability polar carotenoids to exist at the surface of the lipid droplet rendering themselves more available for absorption whereas the non-polar carotenoids reside inside the lipid droplet (18). Following micellarization, carotenoids are transported across the unstirred water layer to be absorbed by the enterocytes of the small intestine. Some studies suggest that carotenoid transport into enterocytes from micelles is active (19,20), whereas other studies show that passive transport dominates at pharmacological doses in rats and Caco-2 cells (21,22). El-Gorab et al. showed that absorption occurred at a nearly linear relation to concentration which led the authors to hypothesize β-carotene uptake occurs by passive diffusion (23). However, this hypothesis was questioned because it did not explain the high variability in carotenoid absorption observed in humans or the competition for absorption observed between carotenoids. This controversy was resolved when a Drosophila gene ninaD encoding the scavenger class B type, I (SR-B1) receptor was identified in 2002 (24). Both in vitro cell culture studies and in vivo studies using mutant mouse models have since established SR-B1, along with Cluster of Differentiation 36 (CD-36) and NPC1 like intracellular cholesterol transporter 1 (NPC1L-1) mediate carotenoid uptake from the lumen into the enterocytes (25–27). Once taken up by the enterocyte, provitamin A carotenoids can be packaged into chylomicrons in their intact form or can be enzymatically cleaved to retinol.

The conversion of provitamin A carotenoids to retinol occurs in two pathways: central and eccentric cleavage. The central cleavage of β-carotene occurs by dioxetane adduct of the central double bond to yield two molecules of retinal catalyzed by the enzyme β-carotene 15-15' – oxygenase (BCO1) (28). Studies by Von Lingtig et al (29) and Wyss et al (30) successfully cloned and sequenced cDNAs encoding enzymes having BCO1 activity from the fruit fly *Drosophila melanogaster*. Both research groups showed that the purified recombinant BCO1 catalyzed exclusively the central cleavage of β-carotene and that it depended on ferrous iron as a cofactor. Von Linting's group provided direct genetic evidence by showing that mutations in the gene encoding BCO1 causes blindness in drosophila mutant ninaB. Subsequently, other investigators identified and characterized the BCO1gene in the mouse and human (31–35). Eccentric cleavage occurs at the peripheral double bonds of the molecule producing a series of β-apocarotenals; long-chained apocarotenals can be further oxidized to retinoic acid. Soon after BCO1 was identified and characterized, Kiefer et al (36) identified a cDNA encoding a second type of cleavage enzyme, β-carotene 9', 10'-oxygenase (BCO2). This enzyme was proposed to catalyze the eccentric cleavage of β-carotene at the 9', 10' double bond. BCO2 was later found to have higher specificity to cleave carotenoids with 3-hydro-ionone rings, like xanthophylls and canthaxanthin with 4-oxo-substituted ring sites (37). Interestingly, Hessel et al (38) found that BCO1-knockout mice that were fed β-carotene had an accumulation of intact β-carotene in several tissues accompanied by decreased vitamin A levels despite the expression of BCO2. This finding suggests that BCO1 is the primary enzyme for vitamin A production and BCO2 may be responsible for different physiological functions. In addition, the expression of BCO2, but not BCO1, in human prostate and endometrial connective tissue, cardiac and skeletal muscle, and the endocrine pancreas, suggests that BCO2 might have a function independent of vitamin A production. Since oxidized

eccentric cleavage products, β-apocarotenoic acids, are analogs of retinoic acid, they have been a subject of recent interest in their role as ligands for retinoid receptors. Evidence of this possibility was provided by Eroglu et al (39). This study showed that β-apo-14-carotenoic acid and β-apo-13-carotenone function as antagonists of retinoic acid receptors, and block the retinoic acid-induced activation of endogenous genes that contain the retinoic acid response elements in their promoters. These results indicate that the eccentric cleavage pathway might play an important role in regulating the transcriptional activity of retinoic acid by a feedback mechanism.

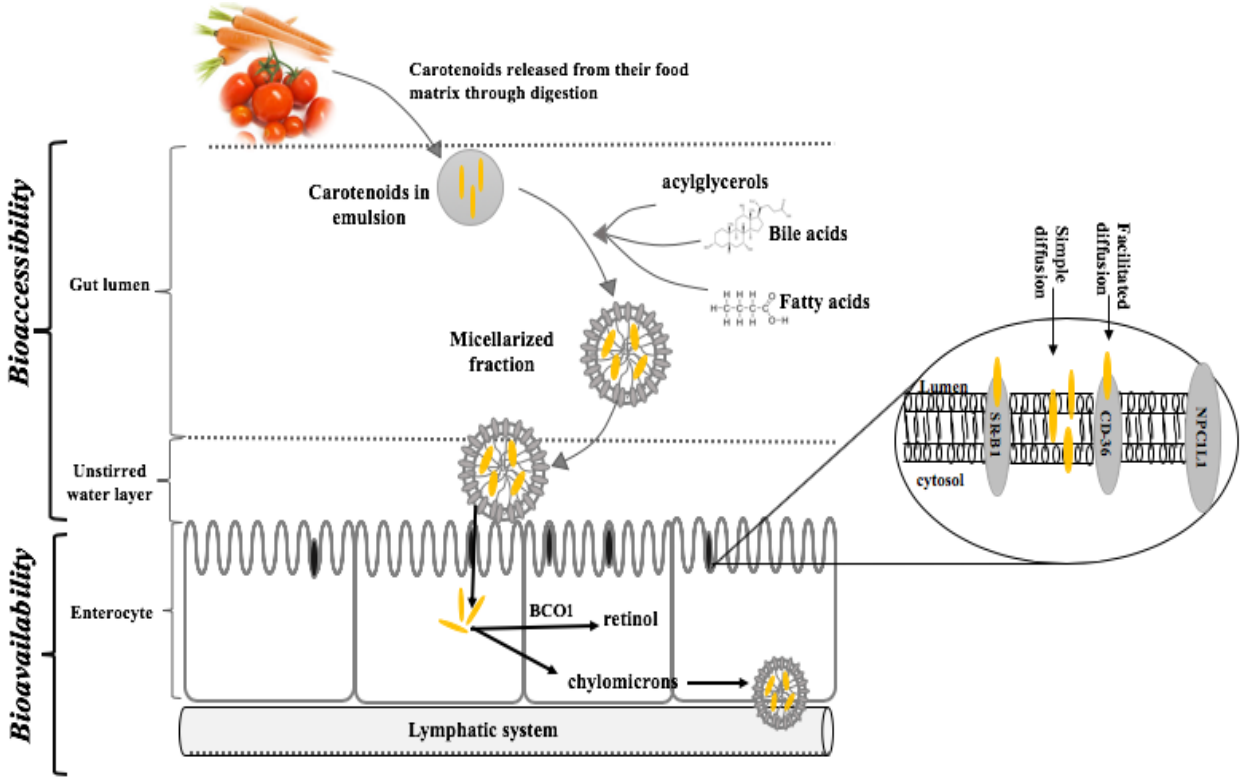

Figure 1.3 Carotenoids are released from their matrix and incorporated into mixed micelles prior to their absorption by the enterocytes. Their absorption occurs through simple diffusion or facilitated by transporter proteins (CD36, SR-B1, NPC1L1)

It is estimated that between 20 to 75% of the ingested provitamin A carotenoid, particularly β-carotene, is converted to vitamin A within the enterocyte (40). The conversion to vitamin A is dependent upon vitamin A status as well as dose of provitamin A carotenoids as it has been shown

The intact provitamin A carotenoids are then packaged into nascent chylomicrons in the Golgi apparatus of the mucosal cells and released into the lymphatic system to be delivered to target tissues. While majority of the conversion of provitamin A carotenoids to vitamin A occurs in the small intestine, the presence of BCO1 in other tissues including the liver, kidney, skin, skeletal muscle and the Retinal Pigment Epithelium (RPE) indicates that carotenoid cleavage is not limited to the enterocyte (41).

While theoretically, a central cleavage of β-carotene molecule yields two molecules of retinol, the actual conversion factor of β-carotene to vitamin A has been shown to be widely variable both from pure β-carotene and provitamin A carotenoid rich plant sources. A review by Haskell showed that vitamin A equivalence of β-carotene from plant sources could range from 3.8:1 to 28:1, by weight (42) highlighting the importance of understanding factors that affect the bioaccessibility and bioavailability of carotenoids in order to estimate their efficiency in conversion to vitamin A. Bioaccessibility is defined as fraction of carotenoids released from the food matrix whereas bioavailability is the fraction of ingested carotenoids available for distribution to tissues for physiological function and storage (**Figure 1.3**) (43). The following sections of the review will primarily focus on provitamin A carotenoids from native African plant materials and factors that affect their bioaccessibility, bioavailability and their ultimate efficacy to meet vitamin A requirements in Sub-Saharan Africa.

1.4 Leveraging native African plant materials rich in provitamin A carotenoids to meet vitamin A requirements in Sub-Saharan Africa

1.4.1 Dietary provitamin A carotenoid sources in Sub-Saharan Africa

As previously stated, fruits and vegetables contribute >80% vitamin A consumption in low-income regions such as Sub-Saharan Africa (44). While Sub-Saharan Africa is believed to be

home to a wide range of indigenous fruits and vegetables potentially rich in micronutrients, information on comprehensive nutrient content of such plants is extremely scarce. Reliable and accessible resources of nutrient content and assessment of dietary consumption patterns are crucial to develop accurate food-based recommendations to tackle vitamin A deficiency. The International Network of Food Data Systems (INFOODS) led by FAO was established in 1984 consisting of food composition experts from around the world in an effort to develop and/or improve regional and national food composition databases in regions like Sub-Saharan Africa. Since the establishment of INFOODS, significant progress has been made in providing accurate resources of food composition in Sub-Saharan Africa although lack of financial support and organizational commitment remain a challenge.

Commonly consumed provitamin A carotenoid-rich plant foods are presented in Table 1-1 based on the West African Food Composition database compiled according to the INFOODS standard guidelines (45). Dark-green leafy vegetables such as moringa (drumstick), amaranth leaves, pumpkin leaves and spinach contain substantial amounts of provitamin A carotenoids (presented as β-carotene equivalent) with levels ranging 1200 – 8850 μg/100g of edible portion. Among the fruits, deep orange flesh mango was considered rich source of provitamin A containing up to 4720 μg/100g followed by néré (2430 μg/100g) and cantaloupe (1900 μg/100g). Root crops such as carrots and deep yellow/orange fleshed sweet potatoes also provide significant amounts of provitamin A carotenoids although the contribution of pale yellow sweet potato is minimal. Red palm oil extracted from the pulp of palm tree nut is also another source of provitamin A carotenoids in this region with levels as high as 68680 μg/100g. The individual provitamin A carotenoid content was not provided in the Food Composition Database and the ones identified in **Table 1.1.** are based on previous reports on carotenoid profile of the specific plants.

While plants rich in provitamin A carotenoids may be available in this region, the consumption pattern of these plants is widely variable across countries in Sub-Saharan Africa. For instance, a 2005 report by WHO on patterns and determinants of fruit and vegetable consumption in Sub-Saharan Africa showed that only about one fifth of all households consumed fruits in countries like Ethiopia, Mozambique and Uganda whereas half of all the households did so in Burundi, Kenya, Malawi, Tanzania and Guinea (46). Additionally, the use these plant materials as vitamin A sources throughout the year is dependent upon geographical location and climate condition. For example, sweet potato is available 11 months of the year and serves as a primary staple crop in countries with two rainy seasons such as Rwanda, Burundi and Uganda whereas in South Africa, where there is only one rainy season, sweet potato is only available 4-8 months of the year (47). Therefore, these factors should be carefully evaluated when developing food-based strategies targeting specific plant sources rich in provitamin A carotenoids for different parts of Sub-Saharan Africa.

Table 1-1 Common provitamin A carotenoid-rich sources in Sub-Saharan Africa and their estimated vitamin A content (μg retinal activity equivalent – RAE) 1,2,3

Food sources	β-carotene equivalent (μg/100g) edible portion	Primary provitamin A carotenoids [2]	Vitamin A value (μg RAE) [3]
Mango (pale - deep orange flesh)	708 - 4720	β-carotene, α-carotene	59 - 393
Papaya	966	β-carotene, β-cryptoxanthin	80
Melon, cantaloupe, raw	1900	β-carotene, α-carotene, β-cryptoxanthin	158
African locust bean (néré)	2430	β-carotene, α-carotene	203
Carrot	8560	β-carotene, α-carotene, β-cryptoxanthin	713
Sweet potato (pale - deep yellow)	39 - 4770	β-carotene, α-carotene	3-397
Pumpkin squash	1200	β-carotene, α-carotene	100
Pumpkin leaves	2300	β-carotene, α-carotene	192
Moringa (drumstick) leaves	8850	β-carotene, α-carotene	738
Amaranth leaves	2890	β-carotene, α-carotene	241
Spinach	6040	β-carotene	204
Red palm oil	68680	β-carotene, α-carotene	5720

[1] Information was gathered from the West African Food Composition database provided by FAO
[2] Primary provitamin A carotenoids are based published articles (not provided by the FAO database)
[3] Vitamin A RAE (μg): Total vitamin A activity expressed in mcg retinol activity equivalent (RAE)= mcg retinol + 1/12 mcg β- carotene + 1/24 mcg α-carotene + 1/24mcg β-cryptoxanthin

1.4.2 Evidence for efficacy of native plants for the treatment and prevention of vitamin A deficiency in Sub-Saharan Africa: pre-clinical studies

Animal models are often considered advantageous to study bioavailability and bioefficancy of provitamin A carotenoids as compared to human studies due their cost effectiveness, the ability to induce deficiency, use radiolabeled compounds and isolate target tissues (48). While several animal models are used in carotenoid studies including rats, mice, ferrets, non-human primates, only pre-ruminant calves and Mongolian gerbils are actually able to convert dietary β-carotene to vitamin A with efficiency similar to humans (49). Nonetheless, information gathered from different animal models has significantly contributed to our understanding of carotenoid metabolism and physiological function.

The use of native African plant materials rich in provitamin A carotenoids has been shown to significantly contribute to vitamin A status in Mongolian gerbils (Table 1-2). A study by Ejoh et al. (2010) demonstrated that relatively small amounts (5.83 nmol vitamin A/g of diet) of provitamin A carotenoid rich plants obtained from Cameroon such as moringa, black nightshade, alpine African bitter leaf and kenaf were able to maintain vitamin A status by significantly increasing liver vitamin A stores as compared to the vitamin A-deficient gerbils (50). However, there were no significant differences between serum retinol levels of treatment groups. Liver vitamin A (retinol) stores are considered the gold standard measure of vitamin A status whereas changes in serum retinol levels are less likely to be accurate in predicting vitamin A status as serum retinol is homeostatically regulated and significant changes are only observed during severely deficient conditions.

Similarly, the use of provitamin A carotenoid rich papaya, orange, tangerine and mango as well as vegetables including spinach, kale and brussels sprouts were also shown to maintain vitamin A status in vitamin A deficient Mongolian gerbils (51). However, banana treatment did

not prevent vitamin A deficiency although measurable amounts of α-carotene and retinol were detected in the liver. Based on differences in total liver vitamin A levels in banana group compared to retinol supplemented group, the conversation factor of β-carotene to retinol was determined to be 28:1 which much higher than the other fruits and vegetables. Papaya and orange were of particular interest as they showed significant increase in liver vitamin A stores as compared to the other fruits. This finding was primarily attributed to the relatively high β-cryptoxanthin content of papaya and orange which has been shown to be more bioavailable and efficacious than β-carotene (52). While tangerine is also known to contain appreciable amounts of β-cryptoxanthin, the lack of increase in liver vitamin A stores (although vitamin A status was maintained), was suggested to be due to impact of food matrix and species variation causing varying bioaccessibility of provitamin A carotenoids. Overall, these studies support the notion that native plants rich in provitamin A carotenoids can significantly contribute to vitamin A status.

Table 1-2 Effect of native plant materials on vitamin A status in vitamin A-depleted Mongolian gerbils [1]

Food source	dose (VA/g diet)	duration of study	biochemical indicators	result	Conversion factor (μg βCE to μg retinol)	Reference
Solanum nigrum (black nightshade)	5·83 nmol	4 weeks	serum and liver retinol, BCOM-1	maintained liver VA status, no difference in serum retinol and expression of BCOM-1	2.1:1	Ejoh et al. (2010)
Moringa oliefera (moringa or drumstick)	5·83 nmol	4 weeks	serum and liver retinol	maintained VA status, no difference in serum retinol	2.3:1	Ejoh et al. (2010)
Vernonia calvoana (Alpine African bitter leaf)	5·83 nmol	4 weeks	serum and liver retinol, BCOM-1	maintained VA status, no difference in serum retinol and expression of BCOM-1	2.1:1	Ejoh et al. (2010)
Hibiscus cannabinus (Kenaf)	5·83 nmol	4 weeks	serum retinol and liver retinol stores	maintained VA status, no difference in serum retinol	1.9:1	Ejoh et al. (2010)
Brassica oleracea var. gemmifer (brussels sprouts)	~5·2 nmol	4 weeks	liver retinol stores	maintained VA status	2.3:1	Arscott et al. (2011)
Brassica oleracea var. acephala (kale)	~5·2 nmol	4 weeks	liver retinol stores	maintained VA status	3.2:1	Arscott et al. (2011)
Spinacia oleracea (spinach)	~5·2 nmol	4 weeks	liver retinol stores	maintained VA status	2.8:1	Arscott et al. (2011)
Banana (cultivar not provided)	~5·8 nmol	3 weeks	liver retinol stores	did not maintain VA status	28:1	Arscott et al. (2011)
Mangifera indica (mango)	~5·8 nmol	3 weeks	liver retinol stores	maintained VA status	2.8:1	Arscott et al. (2011)
Orange (cultivar not provided)	~5·8 nmol	3 weeks	liver retinol stores	increased liver VA stores	1.8:1	Arscott et al. (2011)
Citrus tangerina (tangerine)	~5·8 nmol	3 weeks	liver retinol stores	maintained VA status	3.9:1	Arscott et al. (2011)
Carica papaya (papaya)	~5·8 nmol	3 weeks	liver retinol stores	increased liver VA stores	1.7:1	Arscott et al. (2011)

[1] βCE = β-carotene equivalent calculated as β-carotene + 1/2(α-carotene + β-cryptoxanthin)

1.4.3 Evidence for efficacy of native plants for the treatment and prevention of vitamin A deficiency in Sub-Saharan Africa: clinical studies

Several randomized control trials, intervention and observational studies have been conducted to determine the efficiency of native provitamin A carotenoid rich plant sources to meet vitamin A requirements in different parts of Sub-Saharan Africa (Table 1-3). Results from these studies demonstrate that these plant foods can be leveraged to supply adequate provitamin A carotenoids to improve or maintain vitamin A status in children and women of childbearing age. A randomized controlled trial conducted in Gambia showed that children aged 2-7 years who received 75 g of dried mango with 5g of fat for 4 months had significantly higher serum retinol levels as compared to the control group (40 mg α-tocopherol) (53). Similarly, a study by Van Jaarsveld et al. (2005) demonstrated that children who consumed boiled and mashed orange-fleshed sweet potato for 53 school days showed significant improvement in liver vitamin A stores as compared to the control group (white-fleshed sweet potato) as measure by modified relative dose response (MRDR) (54). The supplementation of biscuits prepared with red palm oil was shown to significantly increase serum retinol levels in South African primary school children (55). Another study in Burkina Faso also showed that the addition of red palm oil (15 ml) to individual meals 3 times a week for a year showed a significant improvement in vitamin A status as determined by serum retinol levels (56). The use of other fruits and vegetables including carrots, butternut squash, amaranth, egg plants, spirulina, and spinach were also reported to improve

vitamin A status both in women and children who are at risk of vitamin A deficiency (57–59). Contrary to these findings, Faber et al. (2016) reported no significant effect in serum retinol levels of subjects who consumed provitamin A carotenoid-rich fruits and vegetables (60). However, it's important to note that the subjects from these studies were not vitamin A deficient based on the cut off for serum retinol level (0.7 μmol/l) and the extent to which these fruits and vegetables can improve vitamin A status cannot be fully captured by serum retinol levels as its highly regulated within the body.

These clinical studies also provide important insight into the impact of matrix of carotenoid source and food processing on the efficacy of these fruits and vegetables to meet vitamin A requirements. For instance, a placebo-controlled trial conducted in Zimbabwe showed that lactating women who consumed either puréed papaya (650 g) or grated carrots (100 g) for 60 days had significantly higher serum retinol concentration as compared to the placebo group (61). However, when liver vitamin A stores were estimated using relative dose response (RDR) method on a subsample of the participants (n=43), the percentage of women with low liver vitamin A stores (RDR > 20%) decreased significantly with papaya but not carrot group as compared to the control. Potential factors for increased hepatic vitamin A stores with puréed papaya but not grated carrots could be due to several factors including differences in carotenoid profile of papaya and carrot, impact of food matrix and amount of test meals provided. Papaya is known to contain higher levels of β-cryptoxanthin which is believed to be more bioavailable than β-carotene (62).

Additionally, the matrix present in the grated carrots may interfere with the release of carotenoids as it has been shown that β-carotene is more bioavailable from carrot juice as compared to raw, chopped carrots (63). Therefore, it is important to carefully evaluate the choice of carotenoid source and different food preparation methods in order to maximize the potential of native plant materials to address vitamin A deficiency in food-based strategies.

Table 1-3 Effect of native provitamin A rich carotenoids plants on vitamin A status in humans [1,2]

Subjects	Study location & design	Sources of provitamin A carotenoids (dose)	Duration	biochemical indicators	Results	Reference
6-12 yr children (n=86/group)	South Africa, parallel-group randomized controlled trial	Dish (300 g) consisting of amaranth, spiderplant, pumpkin and cowpea: no information on carotenoid content	3 months	Serum retinol	no significant effect	Faber et al, 2016
Lactate women (n=50/group)	Zimbabwe, placebo-controlled trial	β-carotene capsules (6 mg), puréed papaya (650 g), grated carrots (100 g)	60 days	Serum retinol	significant increase in serum retinol with beta-carotene group, the papaya group and the carrot group, but not in the placebo group	Ncube et al., 2001
2.5-6 yr children (n=88/group)	Ghana, randomized, double-blind, controlled study	Cassava (*Manihot sp.*) and kapok (*Ceiba sp.*) leaves: 400 RE + fat, or 400 + fat + anthelmintic, or 400 RE + no fat, or 10 RE + no fat	3 months (once/d, 7 d/wk)	Serum retinol	significant increase in serum retinol wtih consumption of dark green leafy vegetables	Takyi, 1999
5–10 yr children (n=90/group)	South Africa, randomized controlled unmasked feeding trial	orange-fleshed sweet potato: 125 g or 1031 µg RAE	53 school days	MRDR test with serum retinol	significant increase in liver vitamin A stores in the treatment group relative to the control group. Significant increase in serum retinol from bseline in both groups	Tanumihardjo , 2005
5-11 yr children (n=146/group)	South Africa, randomized controlled trial	biscuit with red palm oil: 1.23 mg of β-carotene supplied to meet 30% of the RDA	59 school days	serum retinol	significant improvement in serum retinol concentration with treatment group (red palm oil or β-carotene supplement) as compared to the control group	Stuijvengerg et al, 2001
2-7 yr children (n=~44/group)	Gambia, randomized intervention trial	dried mango: 75 g or □150 µg RAE with or without of fat (5 g)	4 months (5 d/wk)	serum retinol and plasma β-carotene	increased plasma β-carotene concentrations, no significant differences were observed at 8 weeks between the mango, vitamin A, and placebo groups in terms of plasma β-carotene or retinol concentrations after adjusting for baseline characteristics	Dremmeh et al, 2002
94 –102 mo infants at baseline (n=128/group)	Burkina Faso, controlled intervention trial	Red palm oil: 15 ml or 1500 µg RAE/meal	12 months (3x/wk)	Serum retinol	significant increase in serum retinol with red palm oil addition	Zeba et al, 2006
Pregnant women (n=30/group)	intervention trial	dark green leafy vegetables with red palm oil or sunflower oil: ~12 g of oil	6 months	α- and β-carotene in plasma and breast milk, serum retinol	red palm oil significant increased of α- and β-carotene in both breast milk and serum and maintained breast-milk retinol concentrations	Lietz et al., 2001
18 - 40 yr women (n=35/group)	Chad, observational	Spirulina: 10.8mg of β-carotene/day		β-carotene and retinol in serum	the serum retinol and β-carotene concentrations were significantly higher in women who consumed Spirulina as compared to those who did not	Soudy et al, 2018
6-12 yr children (n=142)	Ghana, one-group longitudinal pre- and post-intervention	red palm oil bean-stew with gari (280 - 300g cotaining 645 µg β-carotene/100g)	6 months (3x/wk)	Serum retinol	signifiant increase in serum retinol concentration post intervention	Egbi et al, 2017
4-9 yr children (n=51/group)	Ghana, pre-post nutrition intervetion study	stew with or without composite of eggplant and amaranthus leaves : (~498.1 µg β-carotene/day)	3 months	Serum retinol	no significant differences (p = 0.067) in serum retinol concentrations between treatment and control group	Egbi et al, 2018
2.5–6 yr children (n=56/group)	Kenya, intervetion study	cooked dish (80g) containing mixed sun-dried cowpea and amaranth leaves (20:80 wt/wt): 806 µg/g and 599 µg/g β-carotene dry matter, respectively	13 weeks (1/d - 5x/wk)	serum beta carotene and retinol	significant increase in both serum β-carotene and retinol levels with treatment group	Nawiri et al, 2013
~ 13 mo at baseline (n= 498/group)	Mozambique, quasi-experimental intervention study	Orange-fleshed sweet potato median (median 426 vs. 56 µg RAE in control group)	24 months	Serum retinol	significant increase in serum retinol levels in intervention group and no significant change in control group	Low et al., 2007
Postpartum women (n=51)	Ghana, community-based, exploratory intervention trial of postpartum mothers	African eggplant leaves: 200 g containing 2.6 mg of β-carotene	3 months (daily)	MRDR test	significant improvement in vitamin A status with the intervention group	Tchum et al., 2009
2-5yr children (n=107/group)	A home-gardening program was integrated with a community-based growth-monitoring system	Butternut squash, carrots, orange-fleshed sweet potatoes, and spinach	20 months	dietary intake, serum retinol, and growth of children	siggnificant increase in serum retinol concetration in the experimental group	Faber et al, 2002

[1] Abbreviations: MRDR test: Modified Relative Dose Response

[2] Vitamin A RAE (µg): Total vitamin A activity expressed in mcg retinol activity equivalent (RAE)= mcg retinol + 1/12 mcg β- carotene + 1/24 mcg α-carotene + 1/24mcg β-cryptoxanthin

1.5 Challenges to meet vitamin A requirements: bioaccessibility and bioavailability of provitamin A carotenoids

1.5.1 Method of estimation: simulated *in vitro* digestion coupled with Caco-2 cell model

As stated earlier, the efficiency by which provitamin A carotenoids can meet vitamin A requirements is dependent upon several factors which can influence their bioaccessibility (release from their food matrix and incorporation into mixed micelles) and bioavailability (absorption by the enterocytes and delivery to target tissues). Several *in vitro* and *in vivo* methods have been applied to estimate the bioaccessibility and bioavailability of provitamin A carotenoids from different plant sources. *In vitro* methods such as simulated digestion models are particularly useful to study the effect of food matrix and events within the gastrointestinal tract in isolation as it is challenging to do so in humans. Garret et al. (1999) were the first to develop the *in vitro* digestion coupled with Caco-2 human intestinal cell model to estimate carotenoid bioaccessibility based on a previous method developed to study iron bioavailability (64,65). Caco-2 cells have been extensively used to estimate carotenoid absorption due to their ability to undergo spontaneous differentiation and acquire morphological and functional characteristics of enterocytes (66). While the parental Caco-2 cell line (HTB-37) has been used to investigate carotenoid absorption in its intact form, studies concerning carotenoid conversion to vitamin A are usually carried out using the clonal cell line (TC-7) as the activity of the cleavage enzyme BCO-1 is detected in TC-7 and not in HTB-37 (67). Reboul et al. (2006) have demonstrated that these *in vitro* models used for the estimation of carotenoid bioaccessibility significant correlate to bioavailability studies in humans from a wide range of food sources (68). This finding validates the use of such models to efficiently screen numerous samples for the optimal delivery of carotenoids without the cost and complexity of animal and human studies.

1.5.2 Factors that affect bioaccessibility and bioavailability of provitamin A carotenoids

Food matrix: Identifying the form of carotenoid deposition within the plant cells is crucial in understanding bioaccessibility and bioavailability of provitamin A carotenoids. Carotenoids can exist as crystalline aggregates in the chromoplast such as in carrots and tomatoes or in lipid droplets such as mango, papaya and pumpkin. They can also be found the chloroplast of some green leafy vegetables complexed in proteins (69). In vivo and in vitro studies have shown that carotenoids complexed in chloroplasts are far less accessible than the once found in crystalline aggregates or lipid droplets in the chromoplast (70,71). A study by De pee et al examined the impact additional daily portion of dark-leafy vegetables in breast-feeding Indonesian women (70). This study demonstrated that the breast-feeding women who consumed the vegetables for 12 weeks did not show any significant increase in serum retinol. Contrary to this finding, De pee's group carried another study to evaluate if carotenoids are more accessible from fruits than from dark leafy vegetables. In this study, schoolchildren in Indonesia received 2 meals per day consisting of dark leafy vegetables (684 RE/d) or fruits (509 RE/d) for 9 weeks. Interesting, the fruit diet was approximately twice as effective at increasing serum retinol concentrations as were the vegetables. Another study by Haskell et al. demonstrated that although the consumption of sweet potato and spinach increases total vitamin A stores, β-carotene from spinach was more bioavailable than from sweet potato. By using a deuterated-retinol-dilution technique, Haskell's group determined that adults who consumed 750 RE daily as either sweet potato β-carotene or Indian spinach β-carotene had a conversion factor for sweet potato β-carotene to retinol of 13:1 by weight and for Indian spinach b-carotene of 10:1. These studies highlights that the bioaccessibility of carotenoids is highly dependent on their food matrices.

Dietary fat: In addition to the matrix that the provitamin A carotenoids are found in, factors within the diet of an individual also affect the bioaccessibility and bioavailability of carotenoids.

Among the dietary factors, fat has been shown to have a positive effect. A study by Brown et al. demonstrated that ingestion of salads with reduced-fat salad dressing increased appearance of carotenoids in plasma chylomicrons compared to ingestion of salads with fat-free salad dressing (72). They also showed that the ingestion of salads with full-fat salad dressing resulted in increased concentration of carotenoids in plasma chylomicrons than salads ingested with reduced-fat salad dressing. Similarly, Prince et al. (73) evaluated the effect of fat on accumulation of β-carotene in serum and skin in humans. A single 51mg dose of β-carotene given without dietary fat resulted in no detectable change in serum β-carotene. However, the same dose administered with 200 g fat increased serum β-carotene 2.5-fold at 40h. Unlu et al. (74) carried out a study in humans to test the hypothesis that avocado fruit as a lipid source enhances carotenoid absorption to an extent similar to that of an equivalent amount of added pure avocado oil. They demonstrated that avocado addition (half or whole fruit, equivalent to 75 and 150 g avocado, respectively) significantly enhances carotenoid absorption from salsa and salad with no significant difference between avocado fruit and avocado oil. This was the first intervention study to highlight the use of a fruit as a lipid source with carotenoid-rich foods to enhance carotenoid absorption in humans. Although there are several in vitro and in vivo studies that support the significance of lipid in increasing carotenoid absorption, the minimum amount required for optimal carotenoid bioavailability is still controversial. It's been suggested that 5 grams of fat per day is sufficient for optimum absorption, whereas other studies have shown the 3-5 grams of fat per meal is required for significant carotenoid absorption in adults (71,72,75). It has also been demonstrated that the nature of the dietary fat affects carotenoid absorption. A study by Borel et al. (76) showed that chylomicron β-carotene response was reduced when β- carotene is absorbed with medium-chain triglycerides instead of long-chain triglycerides. The authors proposed that the possible reason for above

observation is due to the reduced chylomicrons secretion in response to the ingestion of medium-chain triglycerides.

Dietary fiber: The presence of fiber has also been shown to modify the bioaccessibility and bioavailability of carotenoids. It has proposed that the presence of certain types of dietary fiber might reduce the bioaccessibility of carotenoids by inhibiting micelle formation thereby increasing fecal excretion of bile acids. However, there are some contradictory findings. Rock et al. (77) demonstrated that pectin has an inhibitory effect on human plasma β-carotene response to a single dose of purified β-carotene administered with a meal. In the presence of pectin, the plasma β-carotene concentration was reduced by 42% compared to the control group. Contrary to this finding, Unlu's group demonstrated that the dietary fiber present in avocado fruit (6.8 g fiber/100 g edible portion) had no inhibitory effect on the absorption of carotenoids and 72% of the dietary fiber found in avocado is insoluble consisting of cellulose, pectin and hemicelluloses. This study indicates that the presence of lipids in fiber-rich foods might overcome the proposed negative effect of fiber on the bioaccessibility and bioavailability of carotenoids.

Minerals: Divalent minerals particularly magnesium and calcium have been shown to limit the bioaccessibility of provitamin A carotenoids both from pure and plant sources. A study by Corte-Real et al. (2016) showed carotenoid bioaccessibility was significantly inhibited with the addition of calcium, magnesium and zinc with calcium having the highest impact in limiting micellarization (up to 100% reduction). The same group also demonstrated that bioaccessibility of carotenoids from plant sources were inhibited to varying degree by divalent minerals (78). The proposed mechanism by which minerals limit the bioaccessibility of carotenoids is attributed to their ability bind fatty acids and precipitate bile salts thereby limiting the micellarization of carotenoids. In regards to carotenoid absorption, it has been reported that carotenoid uptake may

be limited by the presence of iron. A study by Bengtsson et al. (2009) reported that the uptake of micellarized β-carotene by Caco-2 human intestinal cells was significantly inhibited by co-incubation with ferrous chloride (30 μM) (79). Although these in vitro studies need to be substantiated with in vivo studies, they provide important insight into the effect of concomitant incorporation of mineral and carotenoid food sources on individual micronutrient bioaccessibility and bioavailability.

Polyphenols: Studies directly investigating the impact specific compounds that belong to different classes of polyphenols on carotenoid bioaccessibility and bioavailability are scarce. Flavanones, specifically hesperetin, hesperidin and naringenin have been shown to modulate carotenoid absorption and metabolism in Caco-2 cells and Mongolian gerbils (80,81). Claudie et al. (2013) demonstrated that uptake of β-carotene (5 μM) and β-cryptoxanthin (5 μM) by Caco-2 cells was significantly increased with the presence of hesperetin (250 μM) and hesperidin (250 μM). The study also showed that although the presence of iron significantly decreased carotenoid uptake, the addition of hesperidin was able to restore carotenoid uptake counteracting the inhibitory effect of iron. This effect was attributed to the metal-chelating activity of hesperidin. A study by Poulaert et al. (2014) also reported that hesperidin increased the activity of intestinal BCO1 in Mongolian gerbils (82). The ability of hesperidin to modulate BCO1 activity was proposed to be related to its action as agonist of PPARγ expression. On the contrary, naringenin was shown to inhibit lutein absorption by Caco-2 cells (80). Taken together, these studies indicate that the modulatory effects of phenolic compounds on carotenoid absorption may be carotenoid species-dependent although additional studies incorporating broader classes of phenolic compounds are warranted.

Host related factors: The extent by which provitamin A carotenoids can meet vitamin A requirements can also be determined by host-related factors such as nutritional status, health, and genetic defects. Vitamin A status has been shown to affect the intestinal conversion of Intestinal conversion of β-carotene to vitamin A. Lietz et al. (83) reported that a negative feedback mechanism regulates the intestinal absorption of β-carotene and its subsequent conversion to vitamin A. Similarly, a study in Filipino children showed that the conversion of plant β-carotene to vitamin A varied inversely with vitamin A status. Animal studies indicate that deficiencies of iron and zinc may also affect bioavailability of provitamin A carotenoids. Decreased mobilization of vitamin A from the liver has been observed in Iron deficient rats (84). Low zinc intake significantly reduced the absorption of β-carotene in rats as determined by lymphatic retinol output (85). The authors suggested that zinc deficiency may limit the expression of retinol-binding protein and interfere with retinol homeostasis. These findings have strong implications in understanding factors that affect the bioavailability of provitamin A carotenoids in developing countries with prevalent micronutrient deficiencies. Genetic polymorphisms also affect the conversion of provitamin A carotenoids to vitamin A. Leung et al. (86) identified two common single nucleotide polymorphisms in the gene encoding BCO1, reducing the conversion of beta carotene to vitamin in UK women. These host-related factors may explain the observed interindividual variability of carotenoid conversion in human populations.

Traditional and commercial food preparation methods: Carotenoid content of plant materials are modified during postharvest handling, thermal processing such as blanching, and cooking, as well as drying and storage. The major cause of carotenoid degradation during storage processing of food is enzymatic as well as non-enzymatic oxidation (87). According to a review by Rodriguez-Amaya (87), fruits and root crops may continue to synthesize carotenoids even after

harvest, given these plant materials are kept intact and not treated in any way that would inactivate the enzymes responsible for carotenoid biosynthesis. In contrast, post-harvest degradation appears to prevail in leafy vegetables especially at high storage temperature. Thermal processing may decrease total carotenoid contents, but at the same time may liberate carotenoids from their food matrices and facilitate their solubilization resulting in an increased carotenoid bioavailability (88,89). However, this is highly dependent on time, temperature and condition of processing. Carotenoids from processed plants foods have also been found to be more bioavailable than their raw forms. A study by Rock et al. (90) demonstrated that the consumption of processed carrots and spinach for 4 weeks increased plasma β-carotene that averaged three times higher than the consumption of these vegetables in the raw form. Additionally, reducing the particle size of carotenoid-rich foods during food preparation (e.g. chopping, grinding, or milling) may also increase carotenoid bioaccessibility by enhancing the release of carotenoids from the food matrix (91). However, there is insufficient data to appraise the impact of home preparation and processing of specific local plant materials especially those found in developing countries. Further research in this area is needed to develop technologies that enhance the delivery of provitamin A carotenoids from traditional food processing methods.

1.6 Conclusion and specific objectives

Evidence from pre-clinical and clinical studies has demonstrated that provitamin A carotenoid-rich plant sources can be a promising strategy to meet vitamin A requirements in Sub-Saharan Africa. However, the extent to which these provitamin A carotenoids can improve and maintain vitamin A status is dependent upon several factors including availability of economical foods for target populations that are widely acceptable. As most food-based strategies support the diversification of diets through the introduction of nutrient dense plant foods, there exists a

potential for use of composite cereal porridges which are viewed as key staples in African communities. This strategy would include formulation of materials rich not only in vitamin A but also iron and zinc, two other key shortfall micronutrients. However, in consideration of the factors impacting carotenoid absorption, blending of such systems leads to the potential for synergistic or antagonistic effects due to the concurrent introduction of specific nutrients. The generation of accurate compositional data for targeted plant materials, inclusive of key potential effectors (phenolics, fiber and minerals) as well as the evaluation of their performance in model cereal product is required to develop appropriate guidance for their use in addressing micronutrient deficiencies. In this regard, food-based strategies should also be designed in a way that could easily be incorporated into existing local food preparation methods without increasing the cost or preparation time. Furthermore, targeting community marketing efforts by collaborating with local suppliers and entrepreneurs will ensure the sustainability of this approach.

To advance these goals, the overall objective of the present work was to investigate the suitability of incorporating micronutrient-dense (iron, zinc and provitamin A) native African plant materials in composite-millet products with a focus on potential for interactions that may adversely impact provitamin A bioavailability. Specific objectives are:

Specific objective 1: Chapter 2 explores the nutritional and chemical composition of nutrient-dense African plant materials particularly, *Adansonia digitata* (baobab), *Moringa Oleifera* (moringa) and *Hibiscus Sabdariffa* (hibiscus) to determine their potential use as sources of important nutrients and identify potential enhancers or inhibitors of provitamin A carotenoid bioavailability as well as other micronutrients such as iron and zinc. **Central hypothesis**: Baobab, moringa and hibiscus will contain specific phenolic compounds and polysaccharides that have been previously identified as modulators of carotenoid and mineral bioavailability

Specific objective 2: Chapter 3 describes the impact of incorporating mineral-rich native plant materials (specifically, baobab) on the bioaccessibility of provitamin A carotenoids from blended cereal based products using in vitro digestion coupled with Caco-2 cells. Considering previous reports showing the negative impact of high mineral content on the formation of mixed micelles thereby reducing the bioaccessibility of carotenoids, there is a potential for similar negative effects with the introduction of baobab particularly due to its high divalent mineral content (i.e. calcium and magnesium). **Central hypothesis**: The addition of mineral-rich baobab will negatively impact the micellarization of provitamin A carotenoids from a blended millet-fruit/vegetable thin porridge formulations thereby limiting their subsequent acute absorption by Caco-2 cells.

Specific objective 3: As the habitual consumption of these underutilized fruits and vegetables is critical for the success of food based strategies, it is important to investigate the long-term effects of introducing such nutrient dense plant materials on provitamin A carotenoid absorption as well as any intracellular changes that may be linked to nutrient uptake that would be reflective of a new paradigm of daily use of these materials compared to current less frequent use. Thus, the final chapter investigates impact of repeated exposure of Caco-2 human intestinal cells to nutrient dense plant digesta (a mimic of repeated consumption) on biomarkers of cellular differentiation as well as absorption of provitamin A carotenoids. **Central hypothesis**: Repeated exposure to aqueous fraction of baobab or moringa generated during simulated digestion will alter carotenoid uptake and cellular differentiation biomarkers of Caco-2 human intestinal cells.

CHAPTER 2. ANALYSIS OF PHYTOCHEMICALS AND POLYSACCHARIDES FROM ADANSONIA DIGITATA L. (BAOBAB), MORINGA OLEIFERA (MORINGA), AND HIBISCUS SABDARIFFA (HIBISCUS) SENEGALESE PLANT INGREDIENTS USING UPLC-DAD, UPLC-ESI-MS/MS AND GC-MS

2.1 Introduction

Sub-Saharan Africa has the highest prevalence of undernutrition in the world (92). Specific to micronutrient deficiency, vitamin A and iron deficiency are the most widely occurring affecting primarily preschool children and women of childbearing age. Among the 190 million children reported to be vitamin A deficient around the world, Sub-Saharan Africa accounts for 48% (5). Similarly, more that 60% of the 800 million preschool children and women reported to have anemia caused by iron deficiency are reported to reside in this region (93). The prevalence of these micronutrient deficiencies remains despite the potential of several indigenous fruits, vegetables and plant based foods that can be sourced regionally to supply target nutrients.

Sub-Saharan Africa is home to an estimated 6,376 indigenous plants of which 397 are known to be vegetables (94). Among these indigenous plants, several micronutrient dense materials exist, however information on full nutrient composition, cultivation practices and human consumption is only available for a fraction of these plants. Furthermore, while there is a history of tradition of use for many these plants in food and medicine, broad dietary application or inclusion as ingredients in nutritional and functional foods has not been well documented. With an increased interest in developing sustainable food-based strategies to combat micronutrient deficiencies, promotion of underutilized native plants as ingredients for staple foods has high potential to close the gap in micronutrient intake.

Three indigenous African plant materials including *Adansonia digitata* (baobab), *Moringa Oleifera* (moringa) and *Hibiscus Sabdariffa* (hibiscus) are gaining significant attention for their quality as food ingredients and for their potential to impact human health through their nutrient density and other functional properties. In particular, these plants are reported to be high potential sources of essential nutrients and that they can be leveraged as natural fortification agents in the development of functional foods in Sub-Saharan Africa. The baobab tree is a deciduous tree commonly found in West Africa as well as in certain parts of central and East Africa (95). Different morphological parts of baobab are utilized for various purposed including as an ingredient for preparation of relishes as well as for medicinal purposes for the treatment of fever, cough, dysentery and microbial infections (96). Studies on nutritional composition have demonstrated that baobab fruit is a rich source of minerals (97,98), phenolic compounds that belong to the class of flavonoids, phenolic acids (99,100) as well as important polysaccharides classified as soluble and insoluble dietary fibers (101). Baobab fruit is also known for its high organic acids content including ascorbic, citric acid and malic acid. These organic acids along with the phenolic compounds are believed to contribute to the reported antioxidant capacity of this fruits flesh and potentially and its health promoting properties (102).

Like baobab, moringa is mainly found in tropical and sub-tropical parts of Sub-Saharan Africa, is traditionally used for nutritional purposes as well as for variety of diseases including bacterial and viral infections, hypertension, inflammation and cancer (103). Leaves of moringa contain high amounts of shortfall minerals including calcium, magnesium, potassium and iron, as well a key shortfall vitamins including vitamin A (in the form of provitamin A carotenoids), vitamin E and C (104). Additionally, considerable amounts of phenolic compounds as well as organic acids have been detected in moringa leaves (105,106). Hibiscus plant is an annual

herbaceous shrub which is also widely grown in West and Central Africa. Commonly characterized by its reddish-purple flower, hibiscus plant is extracted for preparation of hot and cold beverages, treatment of diseases as well as a natural coloring agent during preparation of relishes (107). Studies have shown that hibiscus is an important source of minerals, vitamins and bioactive compounds including phenolics, phytosterols and non-phenolic organic acids (108). Few studies have identified key phenolic compounds that belong to the class of anthocyanin including glycosides of cyanidin and delphinidin that are responsible for the vibrant color of the hibiscus flower (109,110).

Success of food-based strategies leveraging micronutrient dense indigenous plants is dependent on several factors including: (1) access to consistent and sustainable raw materials, (2) establishing the nutritional quality and safety of these materials, (3) development of a value chain for processing of these materials into viable products and ingredients and finally (4) successful adoption of these plant materials by consumers leading to enhanced micronutrient delivery. In this regards, knowledge on nutritional composition of such materials is critical as potential for synergic or antagonistic effects on micronutrient bioavailability are possible. For example, Merwe et al. (2018) reported that incorporation of baobab was shown to enhance iron bioaccessibility from blended-millet based porridge primarily attributed to the high levels of ascorbic acid found in baobab. On the contrary, plants rich in specific phenolic compounds and phytic acid have been shown to limit the bioavailability of non-heme iron (111,112). Similarly, the bioavailability of Provitamin A carotenoids have been shown to be modulated by dietary factors such as soluble and insoluble fiber as well as divalent minerals (113,114). The need for more complete profiling of critical nutritional and chemical composition including select bioactive components (e.g. phenolic and polysaccharide) is critical to the development of mechanistic insights into their potential as

nutritional ingredients. Consequently, the objective of this first study was to investigate the phytochemical composition, particularly focused on carotenoids, tocopherols, phenolic compounds, and organic acids mono and polysaccharide composition of currently available baobab, moringa and hibiscus ingredients in the Senegalese market that are targeted for use in new food product development.

2.2 Materials and methods

2.2.1 Materials

All plant materials were obtained from local suppliers in Senegal in 2016. Plant materials were harvested locally and processed by these suppliers to dry powder forms using traditional (but undocumented) methods in Senegal. Baobab fruit pulp powder was obtained from Free Works Production (Dakar, Senegal Feb, 2016). Dried hibiscus flower and moringa leaf powder were from Maria Productions (Dakar, Senegal, Feb, 2016). Received plant samples were stored at -20°C until further analysis. The intent was to establish the composition of these materials as they would be used in food formulation which may differ from the compositional analysis of fresh materials as the impact of processing could not be determined logistically.

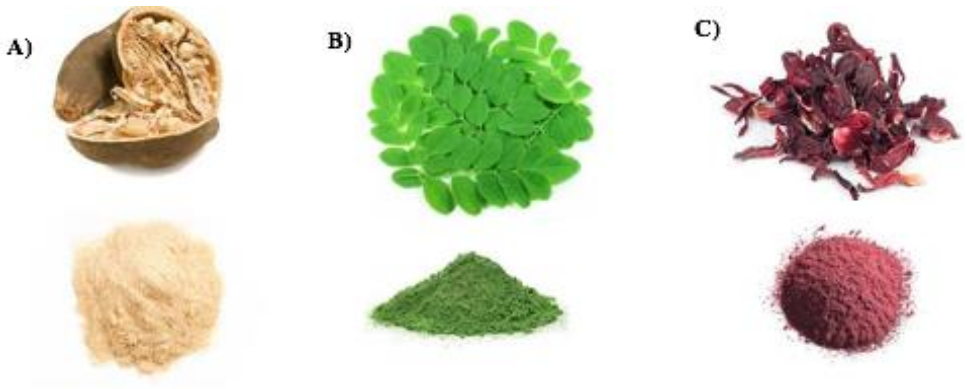

Figure 2.1 Image of A) Adansonia digitata (baobab) fruit pulp, B) Moringa Oleifera (moringa) leaves and C) Hibiscus Sabdariffa (hibiscus) flower. Images were adapted and modified from online sources (115–117)

2.2.2 Carotenoid extraction and analysis

All carotenoid extraction was carried out under a yellow light to minimize potential degradation due to photo-oxidation. Extraction procedure was carried out using method described by Tristan et al. (2013) with minor modifications (118). Briefly, ~ 0.6 g of dried baobab, moringa and hibiscus samples were spiked with 100 µL β-apo-8′- carotenal in petroleum ether (internal standard), rehydrated with 1ml of double-distilled water and placed on ice for 10 min. Samples were then extracted with 5ml of chilled acetone twice followed by 2 ml of methyl tert-butyl ether (MTBE) with BHT (0.1%, w/v). Moringa and hibiscus extracts were further subjected to saponification for the hydrolysis of carotenoid esters and remove potential interference from chlorophyll and lipids during chromatographic separation of target carotenoids. For the saponification process, methanolic KOH (30% v/w) was added to the crude extracts of moringa and hibiscus, incubated for 15 min at 37 °C, washed with saturated NaCl solution followed by an additional wash with double-distilled water. Organic layers were collected and dried under a stream of nitrogen, resolubilized in 0.1% BHT ethyl acetate and methanol (1:1) and filtered through 0.45 µm Polytertrafluoroethylene (PTFE) membrane prior to analysis. Carotenoid

separation and quantification was carried out using Waters Acquity UPLC coupled with photodiode array detector using YMC C30 3 μm 2.0 mm × 150 mm column as previously described (119). Carotenoid all-*trans* isomers and α-tocopherol were determined to be the main tocochromanol in Moringa were identified based on co-chromatography with corresponding authentic standards whereas *cis* isomers and δ-tocopherol and γ-tocopherol were tentatively identified based on UV-vis absorption spectra and comparison to retention times of previous reports in our group (120,121). Quantitation of each *cis* isomers was completed using the response of the all-*trans* form.

2.2.3 Phenolic extraction and analysis

Phenolic extraction was carried out based on the method previously described by Furrer et al. (2017) with slight modification (122). Dried baobab, moringa and hibiscus samples were first defatted using hexane (1:5 w/v). Defatted samples were then reweighed (~ 0.5 g) and extracted using 80% methanol containing 2% formic acid. Samples containing extraction solvent were vortexed for 5 min, centrifuged for 20 min three times. Pulled supernatant was dried under a stream of nitrogen and resolubilized with 0.1% formic acid in water. Extracted samples were further purified using solid phase extraction (Oasis® HLB 6cc (30 mg) extraction cartridges). Once the SPE cartridges were conditions and equilibrated using methanol and water, 1ml of extracted were loaded onto the SPE cartridges and rinsed with 1% FA in water, eluted with 0.1% FA in methanol, dried under a stream of nitrogen and reconstituted with 0.1% FA water and methanol (1:1).

2.2.4 UPLC-MS/MS instrument conditions

LC-MS/MS analysis was conducted using Waters ACQUITY Xevo TQD instrument. Inlet method was optimized for a run time of 6 minutes for 5μl of injection volume. The mobile phase consisted of 0.1% formic acid (v/v) in water (mobile phase A) and 0.1% formic acid (v/v) in

acetonitrile (mobile phase B) at a flow rate of 5 mL/min. The gradient consisted of 0% B at 0 min, 6% B at 0.5 min, 9% B at 2 min, 13% B at 3 min, 35% B at 4.5 min, 0% B at 5.2 min, 0% B at 6 min. Mobile phase A was switched to 2% formic acid (v/v) for the analysis of anthocyanins. ACQUITY UPLC BEH C18 column was used where the column temperature was set at 40 °C. MS source parameters including cone voltage and collision energy were optimized by directly infusing individual standards of target compounds (**Table 2.2**).

2.2.5 Isolation of plant polysaccharides

Isolation of plant polysaccharides from dried baobab fruit, moringa leaves and hibiscus flower was carried out according to Lamothe et al. (2014) with minor modifications (123). A commercial gum arabic from acacia tree (Sigma Aldrich, G9752) was also used in this study for comparison purposes. Dried baobab, moringa, hibiscus and gum arabic were first defatted using hexane (1:7, w/v) twice and left to dry overnight. Defatted plant samples were then suspended in water (1:10, w/v) and pH was adjusted to ~ 7 using 1M $NaHCO_3$. Heat-stable α-amylase (0.2 ml/g of defatted plant samples) (Termamyl A3403, Sigma Aldrich Corp., St. Louis, MO) was added to each reaction tube and incubated in a shaking water-bath (90 rpm) at 80 °C for 1 hour. Samples were then removed from the water-bath, placed on ice and pH was adjusted to 6 with 1M HCl prior to the addition of protease (Neutrase, 0.1 ml/g of defatted plant samples) (P1236, Sigma Aldrich Corp., St. Louis, MO). The liquified suspensions containing the protease were left overnight in a shaking water-bath (90 rpm) at 50 °C. The enzyme-treated suspensions were removed from the water bath and boiled (100 °C) for 15 min to deactivate enzymes. Samples were cooled to room temperature and dialyzed against Mill-Q-water for 24 h using 45-mm diameter Spectra/Por 2 membrane tubing (12-14kDa MWCO) (Spectrum Labs, Rancho Dominguez, CA). Dialyzed samples were then freeze-dried prior to monosaccharide and linkage analysis.

2.2.6 Monosaccharide composition and glycosyl linkage analysis

Neutral sugars were analyzed as alditol acetate derivatives of the glycosyl residues of the polysaccharide isolated from baobab, moringa, hibiscus and gum arabic based on method described by Pettolino et al. (124). Briefly, ~ 0.1 mg of isolated polysaccharide fractions (n=3) were hydrolyzed with 2.5 M trifluoroacetic acid (TFA) for 90 min at 121°C. 100ul of *myo*-inositol (10 mg/ml) was added as an internal standard and samples were dried under a stream of nitrogen. Following hydrolysis, samples were reduced using 1M $NaDB_4$ in 2M NH_4OH by sonicating for 1 min and incubating at room temperature for 2.5 h. Glacial acetic acid was added to remove excess reductants and samples were dried under a stream of nitrogen prior to acetylation. Dried samples were acetylated by using acetic anhydrate where samples were sonicated for 5 min and incubated for 2.5 h at 100 °C. Samples were then washed with distilled water and extracted with dichloromethane (DCM). The DCM layer was dried and resolubilized with acetone prior to analysis using gas chromatography. Analysis of monosaccharides as alditol acetates was carried using GC-FID-MS (7890A and 5975C inert MSD with a Triple-Axis detector, Agilent Technologies, USA) on a capillary column SP-2330 (SUPELCO, USA) with the following conditions: injector volume 1 µl, 240 °C injector temperature, front detector temperature 300 °C, helium as carrier gas, 1.6 ml/min flow rate, 1.16 m/s velocity, 2:1 split ratio; temperature of oven program was 100 °C for 2 min, and then 10 °C/min to 180 °C for 2 min then 4 °C/min to 240 °C for 15 min.

Acidic sugar content of plant polysaccharides were determined by trimethylsilyl (TMS) derivatization according to the method presented by York et al (125). Briefly, ~ 1 mg of polysaccharide fractions were mixed with 1M methanolic HCl and incubated for 16 h at 80 °C to convert the polysaccharides to methyl ester methyl glycosides of uronic acids. The samples are

then silylated using Tri-Sil HTP reagent (ThermoFisher Scientific), incubated at 80 °C for 20 min, dried and resolubilized with hexane prior to analysis by GC (Agilent 6890 series GC-FID) using DB-5 capillary column with the following conditions: 3 min hold of initial temperature at 160 °C, temperature rate of 3 °C/min to 260 °C and 15 min hold. Flow rate was 1ml/min with an injector and detector temperature of 240 and 300 °C respectively.

Glycosyl-linkage composition was analyzed as described by Pettolino et al. (124). ~ 0.1 mg of isolated plant polysaccharides were partially methylated twice with incremental addition of CH_3I following the ionization of hydroxyl groups of the monosaccharide residues using freshly prepared NaOH/DMSO slurry. Subsequently, dried and methylated samples were hydrolyzed using 2 M TFA, reduced with1M $NaDB_4$ and acetylated with acetic anhydrate. Acetylated samples extracted with DCM, dried under a stream of nitrogen and reconstituted with acetone prior to analysis using GC-FID-MS with conditions described in neutral sugar analysis. Linkage points were determined by comparing mass fragmentation pattern of identified peaks with the spectral database for Partially Methylated Alditol Acetate provided by The University of Georgia Complex Carbohydrate Research Center (http://www.ccrc.uga.edu./specdb/ms/pmaa/pframe.html).

2.3 Results and discussion

2.3.1 Carotenoid and tocochromanol profiles of dried baobab, moringa and hibiscus

Analysis of carotenoids and tocopherols of plant samples using UPLC-PDA revealed two major class of carotenoids (xanthophylls and carotenes) as major species in these plant ingredient samples. The carotenes identified in the present study include all-*trans*-β-carotene, all-*trans*-α-carotene, α-cryptoxanthin as well as the cis isomer of β-carotene (9-*cis*-β-carotene). The

xanthophylls – the oxygenated derivates of carotenes – identified include all-*trans*-lutein, zeaxanthin and 9-*cis*-lutein. α-Tocopherol, δ-tocopherol and γ-tocopherol were determined to be the main tocochromanol in Moringa.

Table 2-1 Carotenoid and tocochromanol content in dried baobab fruit, moringa leaves and hibiscus flower a, b

		Concentration (μg/g)		
Carotenoid	**$\lambda_{maxI}/\lambda_{maxII}/\lambda_{maxIII}$**	**Baobab**	**Hibiscus**	**Moringa**
Lutein	445/473	ND	ND	54 ± 4.3
9-cis-lutein	441/470	ND	ND	3.2 ± 0.3
Zeaxanthin	451/477	ND	ND	10.9 ± 0.9
α-cryptoxanthin	447/475	ND	0.3 ± 0	1.7 ± 0.2
all-trans-α-carotene	447/476	trace	trace	1.2 ± 0.1
all-trans-β-Carotene	453/480	0.1 ± 0	0.3 ± 0	16.1 ± 1.7
9-cis-β-carotene	448/480	ND	ND	3.6 ± 0.4
α-tocopherol	292/660	ND	0.2 ± 0	3.1 ± 0.3
***δ*-tocopherol**	298/660	ND	trace	0.1 ± 0
***γ*-tocopherol**	300/660	ND	trace	0.1 ± 0

[a] Data represent the mean ± standard deviation of triplicate plant samples
[b] Abbreviation ND represents Not Detected

Carotenoid and tocochromanol content of plant samples is presented in **Table 2.1**. Generally speaking hibiscus and baobab did not contain meaningful levels of carotenoids or tocopherols. The primary carotenoid in dried baobab fruit was all-*trans*-β-carotene. Levels of 0.1 μg/g found were similar to the low levels reported by Sena et al. (1998) from dried baobab fruit grown in the Republic of Niger (126). Trace levels of all-*trans*-α-carotene was detected in baobab, but these levels were below the limit of quantification. The carotenoids identified in hibiscus flower powder were primarily all-*trans*-β-carotene (0.3 μg/g) and α-cryptoxanthin (0.3 μg/g). α-Tocopherol of hibiscus content was found to be 0.2 μg/g. Most studies reporting

carotenoid content of hibiscus flower have primarily focused on β-carotene content with levels ranging 3 – 34.23 μg/g (107,109,127). Limited information exists on tocopherol content of hibiscus flower as most studies have primarily focused on seed oil extracts and not hibiscus flowers. A study by Mohamed et al (2007) demonstrated that while α-tocopherol was mostly concentrated in the leaf (~ 82 μg/g), substantial amounts were detected in the seed (~ 34 μg/g), stem (~ 7.3 μg/g), and petal (~ 15.5 μg/g) (128).

In contrast to baobab and hibiscus, carotenoid and tocopherol content of moringa leaves were high. all-*trans*-Lutein (54 μg/g) was found to be the predominant carotenoid in moringa followed by all-*trans*-β-carotene (16 μg/g). Considerable amounts of *cis* lutein (3.2 μg/g) and β-carotene (3.6 μg/g) isomers were also detected in moringa indicating potential isomerization that may have occurred during processing (solar or oven drying) of moringa leaves to powder consistent with known effects of thermal processing and light exposure on these compounds (129). The amount of lutein in the present study was in agreement with the study by Kidmose et al (130). However, others have reported levels as high as ten times greater quantity of lutein and β-carotene (131,132). These difference in carotenoid content are hard to fully explain from the current knowledge but likely results from the fact that many of these studies used freshly collected leaves whereas the samples from the current study have undergone several processing stages and storage conditions known to affect carotenoid profiles. Furthermore, differences in geographical location,

growing conditions maturation stage during harvest may contribute to the differences in carotenoid content (118). Moringa leaves also contained meaningful amounts of α-tocopherol (3 μg/g). The level of α-tocopherol determined in the present study was similar to that of the study by Sreelatha et al. (2009) assessed in mature and tender moringa leaves (133). Overall, among the plant materials, moringa leaf powder can be considered to be good source of provitamin A carotenoids. Considering the conversion factor provided by FAO/WHO (total vitamin A activity in μg retinol activity equivalent, RAE = 1/12 μg β-carotene + 1/24 μg other provitamin A carotenoids), a serving of moringa powder (5 g) from the current study could provide ~2.5% of recommended dietary allowance (RDA) for children of 1-3 years of age (134). While this contribution might appear low, optimization of post-harvest processing techniques for better carotenoid retention during commercial production of these plants could increase carotenoid yield as other studies on fresh leaves have reported much higher carotenoid content. In addition to provitamin A carotenoids, the high lutein content of moringa is of growing interest. Lutein and zeaxanthin are known to be the primary components of the macular pigment in humans and known to provide protection against photooxidative effects of blue light (135). Their increased consumption has been associated with lower risks of developing degenerative diseases of the eye including age-related macular degeneration and cataracts (135,136). Considering the increase in age-related macular degeneration (AMD) in Sub-Saharan Africa accounting for about 7% of all causes of blindness

(137), native plant foods rich in lutein and zeaxanthin such as moringa could be leveraged to mitigate the risk of AMD in this region.

2.3.2 Phenolic profiles of dried baobab fruit, moringa leaf and hibiscus flower

In the current study, two major classes of phenolics, (flavonoids and phenolic acids) were analyzed using UPLC-MS/MS and MRM parameters for the identification of these compounds is presented in (**Table 2.2**). For flavonoids, individual subclasses of flavonols, flavones, flavanones, flavan-3-ols and anthocyanins were all identified in samples (**Table 2.3**). Specific phenolic acids were further classified into hydrobenzoic and hydroxycinnamic acids (**Table 2.4**).

Table 2-2 UPLC-MS/MS parameters for the identification of phenolic compounds and organic acids in plant samples

Target compounds	Parent (m/z)	fragments	cone voltage	collision energy	dwell time (sec)	Ionization mode
Phenolic acids						
Hydrobenzoic acids						
4-hydroxybenzoic acid	137	64.9 93.1	26	26	0.006	ES -
procatecheuic acid	153.0243	80.9, 90.9	28	18, 24	0.006	ES -
Gallic acid	169.0243	79.1, 125.0	34	16	0.009	ES -
Salicylic acid	175	89, 115	20	15, 10		ES -
vanillic acid	167	108.1, 152.0	80	16, 12	0.006	ES -
Syringic acid	197.07	122.9, 166.9, 182.0	36	24, 22, 14	0.025	ES -
Hydroxycinnamic acids						
Ferulic acid	193.02	134.0, 177.9	30	18, 12	0.025	ES -
Sinapic acid	222.9866	163.9, 207.9	26	18, 14	0.006	ES -
chlorogenic acid	353.0228	84.9, 190.9	26	40, 18	0.007	ES -
Flavonoids						
Flavonols						ES -
Kaemferol-3-galactoside	447	284	42	18	0.006	ES -
Kaemferol-3-glucoside	447	284	42	18	0.006	ES -
Kaemferol-3-rutinoside	593.5	145.6, 150.9, 285.0	58, 58, 40	30, 18, 18	0.015	ES -
Quercitin-3-galactoside	463.0289	271.0, 300.2	42	40, 24	0.015	ES -
Quercitin-3-glucoside	463.0289	271.0, 300.2	42	40, 24	0.015	ES -
Quercitin-3-rutinoside	609.01	300.2	42	24	0.015	ES -
Myricetin-3-glucoside	479.378	150.9, 178.9, 317.0	42	24, 24, 20	0.009	ES -
Flavones						
Apigenin	269.0166	116.9, 148.6	50	32, 24	0.023	ES -
Flavanones						
Nariginin	271.0266	118.9, 150.9	40	26, 18	0.009	
Flavan-3-ols						
catechin	289.1	109.0, 123.0	33	24, 26	0.006	ES -
epicatechin	289.1	109.0, 123.0	33	24, 26	0.006	ES -
Gallocatechin	304.9828	124.9, 178.9	40	20, 14	0.006	ES -
Epigallocatechin	304.9828	124.9, 178.9	40	20, 14	0.006	ES -
Procyanidin B2	576.5	289.5, 407.0	25	25, 30	0.005	ES -
Procyanidin B5	576.5	289.5, 407.0	25	25, 30	0.005	ES -
Procyanidin C1	865.7	289.0, 577.0	35	40, 25	0.006	ES -
Anthocyanins						
Cyanidin-3-galactoside	449.3906	137.0, 213.0, 287.0	46	54, 52, 20	0.015	ES +
Cyanidin-3-glucoside	449.3906	137.0, 213.0, 287.0	46	54, 52, 20	0.015	ES +
Cyanidin-3-arabinoside	419.3906	287	46	20	0.015	ES +
Cyanidin-3-O-sambubioside	581.503	287	46	20	0.015	ES +
Delphinidin-3-galactoside	464.89	68.9, 229.0, 302.9	100	74, 54, 18	0.019	ES +
Delphinidin-3-glucoside	464.89	68.9, 229.0, 302.9	100	74, 54, 18	0.019	ES +
Delphinidin-3-sambubioside	597.502	302.9	40	18	0.019	ES +
Petunidin-3-Galactoside	479.0015	317	40	22	0.015	ES +
Petunidin-3-Glucoside	479.0015	317	40	22	0.015	ES +
Petunidin-3-sambubioside	611.53	317	40	22	0.015	ES +
Organic acids						
Malic acid	133	71.0, 115.0	24	14, 10	0.006	ES -
Citric acid	191	87.0, 111.0	26	18, 10	0.006	ES -
Quinic acid	191	85.0, 93.0	40	22	0.006	ES -

Flavonoids:

Flavonols: The main flavonols identified in the current study include glycosides of kaempferol, quercetin, and myricetin (**Table 2.3**). Moringa leaves were found to contain the highest amounts of flavonols followed by hibiscus and baobab. Quercetin-3-rutinoside (rutin) was the predominant form identified in all three plant ingredients. These results are in agreement with those of Coppin et al. (2013) who reported that glycosides of quercetin were the primary flavonols in different varieties of moringa leaves found in Senegal with levels ranging 1200 – 1120 mg/100g which is in agreement with the current findings in these plant ingredients (138). Similarly, Fernández-Arroyo et al. (2010) reported rutin (49.5 mg/100g) to be more abundant than the glucoside (14.4 mg/100g) in aqueous extract of hibiscus grown in Guerle, Senegal (139). While moringa leaves also contained the high levels of kaempferol-3-glucoside (31 mg/100g) compared to hibiscus and baobab, kaempferol-3-rutinoside was relatively similar in baobab and moringa. Myricetin-3-glucoside was found in relatively higher amounts in hibiscus (15 mg/100g) but was not detected in baobab. Limited information is available on the flavonol content, particularly on the glycoside conjugates, from baobab fruit. However, few studies have reported the presence of the aglycone forms in which quercetin was found to be the primary flavonol similar to the reports in the current study (99,140).

Overall, flavonols identified in the current study have been reported in a wide variety of fruits and vegetables (141). Quercetin and its glycosylated forms are commonly found in apples, berries, buckwheat leaves and seeds as well as onion-family vegetables (142). Buckwheat is known to contain substantial amounts of quercetin-3-rutinoside (rutin) and its extract has been used to as a source of rutin for medicinal purposes (15). Previous reports of rutin content in buckwheat leaves (2344 mg/100g) corresponds to the level of rutin reported in moringa in the

current study. However, the overall quercetin content of moringa seemed to be markedly higher than other fruits and vegetables commonly known to contain these flavonols such as apples (4 – 11 mg/100g), blue berries (8 -19 mg/100g) and red onions 39 – 192 mg/100g) (143). The levels of flavonols found in hibiscus were also relatively higher as compared to these fruits and vegetables. This finding indicates that moringa and hibiscus could be important sources of flavonols that have been associated with positive health effects.

Table 2-3 Flavonoid content of dried moringa leaves, hibiscus flower and baobab fruit a, b

	Concentration (mg/100g)		
	Moringa	**hibiscus**	**baobab**
Flavonols			
Kaempferol-3-O-galactoside	ND	0.4 ± 0.1	0.5 ± 0.1
Kaempferol-3-O-glucoside	31 ± 2.8	4.5 ± 0.3	1.8 ± 0.1
Kaempferol-3-O-rutinoside	13.3 ± 0.8	3.1 ± 0.2	13.2 ± 0.7
Quercetin-3-galactoside	0.1 ± 0.1	3.4 ± 0.2	0.8 ± 0.1
Quercetin-3-glucoside	1632.7 ± 87.2	51 ± 2.4	tace
Quercetin-3-rutinoside	3675.1 ± 47.9	345 ± 8.7	23.4 ± 0.4
Myricetin-3-glucoside	1.4 ± 0.2	15.4 ± 0.7	ND
Flavones			
Apigenin	0.5 ± 0.1	0.4 ± 0.1	trace
Flavanones			
Naringenin	1.3 ± 0.2	1.3 ± 0.1	trace
Flavan-3-ols			
Monomeric flavan-3-ols			
Catechin	ND	ND	59.9 ± 2.4
Epicatechin	ND	ND	845.4 ± 4.4
Gallocatechin	ND	ND	0.4 ± 0
Epigallocatechin	ND	ND	0.9 ± 0
Oligomeric flavan-3-ols			
Procyanidin B2	ND	ND	55 ± 2.1
Procyanidin B5	ND	ND	73 ± 0.8
Procyanidin C1	ND	ND	200.1 ± 4.8
Anthocyanins			
Cyanidin-3-galactoside	ND	0.4 ± 0	ND
Cyanidin-3-glucoside	trace	17.9 ± 0.9	ND
Cyanidin-3-arabinoside	ND	0.2 ± 0	ND
Cyanidin-3-O-sambubioside	trace	991.9 ± 20.9	ND
Delphinidin-3-galactoside	trace	2.6 ± 0.4	ND
Delphinidin-3-glucoside	trace	11 ± 0.8	ND
Delphinidin-3-sambubioside	trace	971.4 ± 32.5	ND
Petunidin-3-Galactoside	ND	ND	ND
Petunidin-3-Glucoside	trace	4.7 ± 0.1	ND
Petunidin-3-sambubioside	ND	1.6 ± 0.1	ND

[a] Data represent the mean ± standard deviation of triplicate plant samples.
[b] Abbreviation ND represents Not Detected.

Flavones and flavanones: Apigenin and naringenin were the primary flavone and flavanone forms, respectively, identified in these materials. Moringa leaves was found to contain 0.5 mg/100g of apigenin whereas hibiscus contained 0.4 mg/100g. Karthivashan et al. (2013) reported the presence of apigenin in moringa leaf extracts although the amount of apigenin was not quantified (144). Naringenin was found in similar levels in moringa (1.3 mg/100g) and hibiscus (1.3 mg/100g). However, both compounds were absent in baobab fruit. In general, studies characterizing these classes of flavonoids in moringa, hibiscus and baobab are extremely scarce.

Flavan-3-ols: Baobab fruit powder was determined to be a rich source of flavan-3-ols. Among the monomeric flavan-3-ols, epicatechin (845 mg/100g) was found to be the most abundant compound in baobab fruit. Catechin (60 mg/100g) was also found in relatively appreciable amounts whereas lower amounts of gallocatechin (0.4 mg/100g) and epigallocatechin (0.9 mg/100g) were detected. Lower amounts of epicatechin (79 mg/100g) but higher amounts of catechin (75 mg/100g) were reported by Irondi et al as compared to the current study (145). In addition to the monomeric flavan-3-ols, three oligomeric flavan-3-ols (procyanidin B2, B5 and C1) were identified in this study. This finding was in agreement with the study by Shahat (2006) elucidating the flavan-3-ol composition of baobab fruit using LC-MS and NMR spectrometry (100). While Shahat's group did not report quantitative data, they demonstrated that epicatechin, procyanidin B2, B5 and C1 were there primary flavan-3-ols found in methanolic extracts of baobab fruit collected from Cairo, Egypt.

The monomeric and oligomeric flavan-3-ols reported in the present study are typically found in grapes, different types of tea, wine, cocoa beans, apples. The epicatechin levels of baobab were within similar range of those levels previously reported in green (10 - 1700 mg/100g) and black tea (40 – 700 mg/g) as well as cocoa powder (200 – 1500 mg/100g) (146–149). Procyanidin

B2 content of baobab seemed to be comparable to previous reports from pure cocoa powder (13 - 57 mg/100g) whereas the procyanidin C1 levels were almost ten times higher as those found in the pure cocoa powder (5 -36 mg/100g) (147). However, procyanidins from defatted cocoa beans have been reported to be markedly greater with levels as high as 1007 mg/100g of procyanidin B2 and 1019 mg/100g of procyanidin C1(150). Procyanidin B5 in baobab was also found to be much higher than fruits commonly known to contain this flavan-3-nol including apples (4 – 5 mg/100g), blueberries (1 – 6 mg/100g, DW) and plum (0.2 – 1.3 mg/100g, DW) (151).

Anthocyanins: Glycosylated cyanidin, delphinidin and petunidin were the main anthocyanins identified in hibiscus and to a small extent, moringa. Delphinidin-3-sambubioside (971 mg/100g) and cyanindin-3-sambubioside (991 mg/100g) were found to be the predominant anthocyanins calculated as procyanidin-3-glucoside equivalents. Petunidin was found primarily as the glucoside form (4.5 mg/100g) although relatively lower amounts of petunidin-3-sambubioside (1.6 mg/100g) were detected. These results are in agreement with previous reports of the anthocyanin profiles of hibiscus flower (110,152,153). The major anthocyanins (delphinidin-3- and cyanidin-3- sambubioside) in hibiscus are not as ubiquitously found as other conjugated forms of these flavonoids. The content of Cyanidin-3-sambubioside in hibiscus from the current study seemed to be substantially higher than a previous report of this anthocyanin in redcurrant (24 mg/100g DW) while other fruits such as black elderberry (1155 mg/100g DW) seemed to have comparable amounts as the levels found in Hibiscus (154,155).

Table 2-4 Phenolic acid content of dried moringa leaves, hibiscus flower and baobab fruit a, b

	Concentration (mg/100g)		
	Moringa	**hibiscus**	**baobab**
Hydroxybenzoic acids			
4-hydroxybenzoic acid	113.7 ± 1.9	3.8 ± 0.3	4.9 ± 0.2
Protocatechuic acid	12.7 ± 0.7	3.7 ± 0.6	1.7 ± 0.2
Gallic acid	22.1 ± 0.9	168.6 ± 7.1	3.4 ± 0.3
Salicylic acid	trace	1.4 ± 0.1	ND
Vanillic acid	trace	17.6 ± 1.4	trace
Syringic acid	trace	6.8 ± 1.1	trace
Hydroxycinnamic acids			
Ferulic acid	1.7 ± 0.2	1.6 ± 0.1	0.9 ± 0
Sinapic acid	trace	2.1 ± 0.1	ND
Chlorogenic acid	126.5 ± 10.1	935.4 ± 10.3	1.3 ± 0.4

[a] Data represent the mean ± standard deviation of triplicate plant samples.
[b] Abbreviation ND represents Not Detected.

Phenolic acids:

Hydroxybenzoic acids: Six hydroxybenzoic acid derivatives were identified in plant materials including 4-hydroxybenzoic acid, procatecheuic acid, gallic acid, salicylic acid, vanillic acid and syringic acid (**Table 2.4**). Among these hydroxybenzoic acids, the most abundant compound detected in moringa leaves was found to be 4-hydroxybenzoic acid (113 mg/100g) whereas protocatechuic acid (13 mg/100g) and gallic acid (22 mg/100g) were found in lower amounts. Similar findings of gallic acid content of moringa leaves grown in Mexico were reported by Valdez-Solana et al. (2015) while a study by Fitri et al. (2011) showed a higher content of hydroxybenzoic acids from moringa leaves collected in Indonesia (105,156). The differences in hydroxybenzoic acid content might be due to potential degradation of flavonols from moringa powder formed during processing as it has been demonstrated that quercetin and rutin are highly susceptible to thermal degradation resulting in hydrobenzoic acid formation (157). Additionally, variety of moringa, seasonal differences or geographical location may contribute to the differences

observed in phenolic acid profile (158,159). All six compounds were detected and quantified in hibiscus flower with gallic acid (169 mg/100g) being the most abundant compound. This finding was in agreement with the study by Ramirez-Rodrigues et al. (2011) showing gallic acid as the predominant hydroxybenzoic acid in hibiscus (160). Similar to moringa leaf content, the three hydroxybenzoic acids detected and quantified in baobab fruit include 4-hydroxybenozic acid (4.9 mg/100g), protocatechuic acid (1.7 mg/100g) and gallic acid (3.4 mg/100g).

Hydroxycinnamic acids: Ferulic acid, sinapic acid and chlorogenic acid were the main hydroxycinnamic acid derivatives identified in plant materials. The ferulic acid content of hibiscus (1.6 mg/100g), moringa (1.7 mg/100g) and baobab (0.9 mg/100g) seemed to be relatively similar. Sinapic acid was found in quantifiable amounts only in hibiscus flower (2.1 mg/100g). Hibiscus flower (935 mg/100g) also contained the highest amount of chlorogenic acid followed by moringa leaves (127 mg/100g) whereas baobab fruit content of chlorogenic acid was found to be 1.3 mg/100g. While the chlorogenic acid content of moringa leaves from the current study were similar to that of Verma et al. (2009), the same study reported higher levels of ferulic acid (12.8 mg/100g) than the current study. The relatively higher amounts of chlorogenic acid found in hibiscus could be of particular interest as this phenolic acid is believed to be one of the major compounds responsible for the positive health effects associated with coffee and other fruits and vegetables (161,162). While the reported levels of chlorogenic acid found in coffee beans (6100 – 6900 mg/100g) are much greater than the levels in hibiscus (163). However, the content in hibiscus appeared to be at a similar or higher level than other common sources of chlorogenic acid including potatoes (2.6 – 383 mg/100g) and blueberries (86 – 131 mg/100g) (164,165).

Table 2-5 Organic acid content of dried moringa leaves, hibiscus flower and baobab fruit a, b

	Concentration (mg/100g)		
Compound	**Moringa**	**hibiscus**	**baobab**
Malic acid	501.9 ± 21.3	2.5 ± 1.1	95.8 ± 18.8
Citric acid	310.1 ± 26.1	14.1 ± 2.7	349.6 ± 61.2
Quinic acid	170.1 ± 10.8	1288.4 ± 11.5	trace

[a] Data represent the mean ± standard deviation of triplicate plant samples.
[b] Abbreviation ND represents Not Detected.

Organic acids: The content of malic acid, citric acid and quinic acid from moringa leaves, hibiscus flower and baobab fruit was evaluated (**Table 2.5**). Malic acid was the most abundant acid in moringa leaves (502 mg/100g) whereas hibiscus leaves (2.5 mg/100g) had the lowest amount. Baobab fruit also contained appreciable amounts of malic acid (96 mg/100g). Citric acid was the highest in baobab fruit (350 mg/100g) followed by moringa (310 mg/100g). Quinic acid in baobab fruit were not quantifiable whereas larger concentrations were detected in hibiscus (1288 mg/100g) and moringa (170 mg/100g). Generally, all three plants seemed to be good sources of organic acids that have been associated with improved iron absorption (166). Additionally, citric acid has been shown to promote thermal stability of β-carotene in emulsions (167).

Collectively, the phenolic profile of the moringa, hibiscus and baobab from the present study could provide important insight into to the vast medicinal and nutritional use of these plant materials. The health benefits associated with the flavonoids and phenolic acids identified in these plants include antioxidative, anti-inflammatory, antimutagenic, anti-microbial, cardioprotective, anti-carcinogenic activity, diabetes and obesity prevention (168–170). In regards to potential antinutritive effects, polyphenols are generally believed to limit iron bioavailability due to their ability to chelate metals (171). Interestingly, recent studies have reported that this inhibitory effect was found to be compound-specific (171,172). Hart et al. (2015) demonstrated that iron uptake by

Caco-2 cells was promoted by kaempferol, kaempferol 3-glucoside, and epicatechin and inhibited by myricetin, myricetin 3-glucoside, quercetin, and quercetin 3-glucoside. Although the potential mechanism by which these specific phenolic compounds promote iron uptake was not elucidated, it was proposed that they might play a role in facilitating the reduction of Fe^{3+} to Fe^{2+} thereby promoting iron uptake. While these findings need to substantiated with *in vivo* studies, it could be predicted that the high levels of epicatechin in baobab could be leveraged to be used as a potential enhancer of iron absorption in product formulation whereas introducing moringa in such formulations may present inhibitory effects due to its high quercetin levels.

In addition to the effect of specific phenolics on iron bioavailability, these compounds also might play a role in the delivery of lipid-soluble provitamin A carotenoids. Flavan-3-ols and other classes of flavonoids are known to play a role in management of obesity and diabetes by modulating carbohydrate and lipid metabolism (170,173,174). A study by Sugiyama et al. (2007) showed that apple flavan-3-ols, particularly oligomeric fractions, significantly inhibited lipid absorption by inhibiting pancreatic lipase activity in mice and in human (175). While this might be a plausible strategy to address hyperlipidemia thereby preventing obesity, deficiencies of fat soluble vitamins remains a concern with such type of treatment. Consequently, flavan-3-ols from baobab should be carefully evaluated for their impact on the bioavailability of vitamin A and carotenoids as their potential inhibitory effect on pancreatic lipase may limit the incorporation of these fat soluble compounds into mixed micelles and their subsequent absorption by the small intestine. Overall, the phenolic profile of the plant materials identification of these compounds could be used to optimize the use of commercially available hibiscus, baobab and moringa powder targeted for specific nutritional and functional benefits.

2.3.3 Monosaccharide and glycosyl-linkage composition of polysaccharide fraction of dried baobab fruit, moringa leaves and hibiscus flowers

The monosaccharide composition of isolated cell wall fractions of gum arabic, baobab, moringa and hibiscus is presented in **Table 2.6**. Commercial gum Arabic powder (Sigma Aldrich, G9752) was simultaneously subjected to alditol acetate derivation along with all plant samples to confirm repeatability across isolated cell wall fractions. The monosaccharide composition of gum Arabic from the current study was found to be similar to previous reports by William et al. (1993) confirming proper derivatization and analysis (176). The polysaccharide composition of baobab fruit was mostly composed of xylose (46%) followed by glucose (16%) and galactose (13%). Baobab also contained relatively low amounts of rhamnose (8%) and arabinose (7%) as well as the uronic acids: galacturonic (2%) and glucuronic acid (3%). Hibiscus flower was primarily composed of galactose (27%), glucose (26%) and xylose (20%). Uronic acids constituted ~2% of the total monosaccharide content with glucuronic acid making up the majority of the uronic acid content in the hibiscus leaf. Moringa leaf was predominately composed of galactose (37%), followed by glucose (23%) whereas rhamnose, arabinose and xylose were found in similar molar ratios. Similar to the hibiscus cell wall fraction, uronic acids in moringa contributed ~2% of the total monosaccharide composition.

Table 2-6 Monosaccharide (mol.%) of baobab, moringa and hibiscus polysaccharide a,b

	Rhamnose	Fucose	Arabinose	Xylose	Manaose	Galactose	Glucose	Galacturonic acid	Glucuronic acid
Baobab	7.0 ± 0.1	trace	7.7 ± 0.1	46.8 ± 0.6	5.5 ± 0.0	13.4 ± 0.1	15.7 ± 0.1	1.8 ± 0.0	2.9 ± 0.0
Hibiscus	9.9 ± 0.1	1.5 ± 0.0	7.5 ± 0.0	20.4 ± 0.2	7.3 ± 0.1	26.6 ± 0.3	24.9 ± 0.3	0.6 ± 0	1.3 ± 0
Moringa	11.5 ± 0.2	1.14 ± 0.0	10.2 ± 0.1	11.0 ± 0.1	4.9 ± 0.0	36.1 ± 0.4	22.6 ± 0.2	0.8 ± 0	1.7 ± 0
Gum arabic	16.1 ± 0.2	trace	27.2 ± 0.3	trace	trace	54.9 ± 0.5	trace	trace	0.3 ± 0

[a] Values represent the mean ± standard deviation of triplicate plant samples and are proportions of total partially methylated alditol acetates.
[b] Abbreviation ND represents Not Detected.

While there is very limited information on neutral and acid sugar composition of these native plant materials using analysis methods described above, some studies have reported monosaccharide composition from different morphological parts of these plants. A study by Woolfe et al (1976) reported that the mucilage extract of baobab leaves from Legon, Ghana were composed of uronic acids, galactose, rhamnose, glucose and arabinose (23:1:0.6:0.4:0.1) as assessed by thin layer chromatography and colorimetric assay (177). Brunold et al. (2002) reported that different fractions of hibiscus flower extract separated by ion-exchange chromatography on DEAE-Sephacel produced varying molar ratios of monosaccharides where water eluate predominantly contained arabinose (78%) and galactose (19%). 0.1 N NaPP eluate contained 36% uronic acid, 23% galactose, 16% glucose, 14% arabinose, 7% rhamnose, 2% xylose, 2% mannose. Increasing the concentration of NaPP (0.1 to 0.5 N) resulted in increased amount of uronic acids and decreased amount of neutral sugars highlighting the impact of polysaccharide isolation method for the characterization of monosaccharides (178). Monosaccharide composition analysis of moringa leaves by He et al. (2018) indicated the presence of rhamnose, arabinose, and galactose in a molar ratio of 1:7.32:12.12 (179). Similar to our findings, uronic acid constituted 2.5% with primarily composed of galacturonic acid.

2.3.4 Glycosyl linkage analysis

Table 2-7 Glycosyl-linkage composition (mol.%) of baobab, moringa and hibiscus polysaccharide a, b

Monosaccharides	linkage	Gum arabic	Moringa	Hibiscus	Baobab
Rhamnose	Terminal-Rham	16.05	3.87	ND	ND
	1--->2 Rha	ND	1.98	2.75	2.34
	1--->2,4 Rha	ND	6.04	7.46	4.95
Arabinose	Terminal-Ara-f	16.94	7.77	3.68	3.97
	1--->4-Ara-p/5-ara-f	ND	2.84	4.12	4.05
	1--->3 Ara	10.29	ND	ND	ND
Xylose	Terminal-xyl	ND	6.18	10.71	26.37
	1--->4-xyl	ND	5.26	10.41	22.32
Mannose	1--->2 Man	ND	5.08	7.59	5.70
Galactose	Terminal-Gal	10.76	6.14	3.44	8.96
	1--->3-Gal	ND	6.20	5.39	ND
	1--->2-Gal	ND	8.52	1.85	4.97
	1--->6 Gal	5.21	ND	ND	ND
	1--->4,6-Gal	ND	11.15	15.89	ND
	1--->3,6-Gal	23.49	5.48	ND	ND
	1--->3,4,6-Gal	15.4	ND	0.94	ND
Glucose	Terminal-Glc	ND	3.67	0.86	0.42
	1--->4-Glc	ND	19.20	24.08	13.69
	1--->4,6-Glc	ND	0.00	ND	2.25
	1--->2,4,6-Glc	ND	0.63	0.83	ND

[a] Values represent the mean ± standard deviation of triplicate plant samples and are proportions of total partially methylated alditol acetates.
[b] Abbreviation ND represents Not Detected.

The glycosyl-linkage composition of plant materials is presented in **Table 2.7**. In baobab fruit, the primary monosaccharide xylose was found to be (Terminal →) and (1 → 4)-linked which were both found in similar mol.(%) ratio indicating sufficient methylation during derivatization (124). (Terminal →)-linked xylose residues along with (1→ 4)- and (1→ 4,6)-linked glucose identified in baobab were attributed to xyloglucans as they are characterized by linear backbone of (1→ 4)-linked glucose residues containing (Terminal →)-linked xylose residues as side chains with some of the xylose side chains substituted with (Terminal →)-linked galactose, arabinose or fucose (180). Xyloglucans are the predominant hemicellulosic polysaccharides of dicotyledons constituting 20-25% of the primary cell wall (180,181). Xyloglucans from Tamarind seeds have been thoroughly investigated for their wide range of applications in the food and pharmaceutical industry including thickening agent, ice-crystal stabilizer, gelling agent and starch modifier (182). These polysaccharides form thermoreversible gels with the presence of high sugar concentrations, alcohols and phenolic compounds particularly flavan-3-ols as crosslinkers and are known to exhibit Newtonian flow behavior at low concentrations and non-Newtonian flow at high concentrations (183–185). Consequently, the traditional use of baobab fruit as a thickening agent might be explained by the presence of high proportions of xyloglucans in this fruit. In addition to xyloglucans, other hemicellulosic polysaccharides in baobab fruit such as heteroxylans were tentatively identified due to the presence of (1 → 4)-linked xylose and (Terminal →)-linked arabinose residues in baobab fruit. Among the pectic polysaccharides, rhamnogalacturonan I (RG-I) was the major polysaccharide predicted to be present in baobab fruit based on the linkages identified. While linkages of uronic acids were not determined in the current study due to their low content (<2%), (1 → 2)-linked and (1 → 2,4)-rhamnose residues found in baobab fruit were

attributed to RG I as they are known to be the major constituents of RG I backbone along with (1 → 4)-linked galacturonic acid residues.

Similar to baobab fruit, (1→ 4)-linked glucose residues and (Terminal →)-linked xylose residues detected in moringa and hibiscus leaves were attributed to xyloglucans although the proportions of these polysaccharides were lower in hibiscus and moringa as compared to those found in baobab. The major pectic polysaccharides tentatively identified in moringa and hibiscus leaves were Type I and II arabinogalactan (AG-I and AG-II) and RG-I. AG-I was structurally identified in moringa and hibiscus by the presence of (1→ 4,6)-linked galactose residues along with (Terminal →)-linked arabinose residues whereas (1→ 3)- , (1→ 3,6)-, (Terminal →)-linked galactose residues and (Terminal →)-arabinose and rhamnose residues were tentatively assigned to AG-II as previously described by Pettolino et al (124). Previous reports have also shown similar glycosyl-linkages in moringa leaves resulting in the identification of arabinogalactans (179,186). AG-II is usually found associated with proteins primarily composed of alanine, serine, threonine and proline/hydroxyproline and is collectively identified as arabinogalactan proteins (AGP) (187). The most common source of AGP is gum arabic, widely utilized for its emulsifying property as its highly hydrophobic protein-rich component is known to preferentially adsorb onto the surface of oil droplets rendering strong emulsion stability (188). Although AG-II-protein complexes were not analyzed in the current study, the high proportions of AG-II found particularly in moringa indicate the potential of such complexes to be present in this plant. This requires a more detailed compositional analysis.

To better visualize the overall composition of these plant materials, major classes of polysaccharides (hemicellulosic and pectic) were estimated according to the mol. % ratio of

linkages assigned for each identified polysaccharide as previously described (123,124). Baobab polysaccharide was estimated to be 54% hemicellulosic (54% xyloglucans) and 7% pectic polysaccharides (RG I). Heteroxylans (~26) tentatively identified in baobab could also be classified under hemicellulosic polysaccharides although their identification cannot be carried out high degree of certainty as linkages of uronic acids were not determined. In hibiscus, pectic polysaccharides were estimated to constitute 39% (16% type I AG, 13% type II AG, and 10% RG I) and hemicellulosic polysaccharides were 40% (xyloglucans) although these polysaccharides might be overestimated as (1→ 4)-linked glucose residues assigned for xyloglucans are also found in cellulose and starch. Moringa polysaccharides were found to be predominantly pectic (60%), primarily composed of type II AG (41%) whereas hemicellulosic polysaccharides (xyloglucan) were estimated to constitute 32%. It is very well stablished that both hemicellulosic and pectic polysaccharides from plant cell walls are associated with several health benefits including gut barrier function, lipid metabolism, glycaemic and insulin responses, reduced risk of colon carcinoma and positive effects on gut microbiota (189). However, their impact on micronutrient digestion and absorption needs to be taken into careful consideration for their use as functional and nutritional ingredients. Dietary fibers such as pectin have been shown to interfere carotenoids bioavailability by potentially entrapping bile salts and increasing their excretion thereby reducing the emulsification of carotenoids prior to their absorption (113,190). In relation to the present study, both xyloglucans and pectic polysaccharides could be predicted to negatively affect the micellarization of carotenoids by modulating the rheological properties of the digesta as well as limiting the accessibility of bile salts. However, further experiments are required to validated this assumption and the extent to which individual polysaccharides may modulate carotenoid bioavailability needs to be determined. In addition to carotenoids, some studies have reported that

dietary fibers could limit iron bioavailability by potentially entrapping or directly binding iron although these findings are not conclusive as others have reported positive association with dietary fiber intake and iron bioavailability (191,192). Nonetheless, different food processing techniques and treatments such as the addition of enzymes targeting inhibiting polysaccharides could be implemented to optimize the use of these plant materials.

2.4 Conclusion

The present study characterized key phytochemical and polysaccharide components of commercially available Senegalese baobab, moringa and hibiscus powders. Moringa leaves were found to be rich sources of carotenoids particularly lutein, zeaxanthin and β-carotene whereas carotenoid content of hibiscus and baobab were relatively minimal. Phenolic composition analysis showed that hibiscus was primarily composed of anthocyanins. Baobab was found to be high in flavan-3-ols whereas moringa and hibiscus were devoid of these classes of flavonoids. In terms polysaccharides, xyloglucans seemed to contribute significant portions of the polysaccharides found in baobab as well as hibiscus. Moringa was predominantly composed of pectic polysaccharides such as type I and type II AG as well as RG I which were also found in hibiscus with lower proportions. While the phytochemical and polysaccharide composition of these plant materials are consistent with their use as traditional health/medical use, the present study also highlights potential for negative impacts between specific phytochemicals (phenolics) and polysaccharides that may interfere with the delivery of target micronutrients (iron and carotenoids) when used as functional and nutritional food ingredients.

CHAPTER 3. AFRICAN ADANSONIA DIGITATA L. (BAOBAB) MODIFIES BIOACCESSIBILITY BUT NOT INTESTINAL UPTAKE OF PROVITAMIN A CAROTENOIDS FROM COMPOSITE FRUIT, VEGETABLE AND MILLET PORRIDGES AS ASSESSED BY IN-VITRO DIGESTION COUPLED WITH CACO-2 CELL CULTURE MODEL

3.1 Introduction

Deficiency of vitamin A, iron and zinc, remains one of the most prevalent public health challenges in Sub-Saharan Africa (193). An estimated 190 million preschool-aged children are affected by vitamin A deficiency of which Sub-Saharan Africa accounts for about 48% of all reported cases (5). Strategies to address this nutritional challenge include supplementation, diet diversification and fortification through traditional or biofortification routes. Many traditional diets in these regions are composed of staples including grains and starch rich foods (cassava and potato) that make up ~60-70% of the calories. However, these foods are typically low in shortfall micronutrient including iron, zinc and vitamin A (4,5). In this context, diet diversification and bio/fortification strategies that leverage local agricultural commodities are increasingly being considered and in some cases implemented as potential sustainable strategies that can simultaneously combat multiple micronutrient deficiencies (194).

The primary sources of vitamin A in Sub-Saharan Africa are provitamin A carotenoids derived from locally available fruits and vegetable (195). Carotenoids, a class of lipid-soluble plant pigments abundant in in dark green, orange and red plant foods, have been associated with many health benefits including reduced risk of several chronic and degenerative diseases (196). However, for the subclass of provitamin A carotenoids, vitamin A activity remains the most critical biological activity in humans. Among the 600+ carotenoids identified in nature, three main forms common in the human diet have provitamin A activity including β-carotene, α-carotene, and β-cryptoxanthin (197). Common sources of provitamin A carotenoids in the African diet include carrots, mangoes, papayas, orange-fleshed sweet potato flesh and leaves, pumpkin leaves, squash and red palm oil (198,199). Several of these materials have been investigated for their efficacy to provide sufficient vitamin A either as individual ingredients or as components of blended foods and diets. (53,54,56,61,200).

While promising, these fruit and vegetables are highly perishable and know to be subject to high post-harvest losses leading to a situation of limited year round availability (201). One possible solution is to develop provitamin A rich ingredients form these materials and incorporation of these materials into staple foods. However, to date, only limited information is available on their efficacy in the context of processed food products, such as extruded porridges, that are growing in prevalence in sub-Saharan Africa. Similarly, indigenous plants being leveraged for minerals in this region include baobab, moringa, amaranth, cowpea, moringa, pumpkin seed, and spider flower (202–204). Baobab (*Adansonia digitata)* is of particular interest as it is not only rich in minerals but also in ascorbic acid which is known to enhance the bioavailability of iron (102,205). A recent study by Merwe et al. (2018) demonstrated that the addition of baobab in a pearl millet porridge co-formulated with provitamin A carotenoid rich carrot and mango significantly increased iron and zinc bioaccessibility as assessed by dialysability assay. While this findings shows promise for leveraging baobab fruit as an enhancer of mineral bioaccessibility, the impact such nutrient dense plant material on the delivery of carotenoids from blended cereal products has not been investigated.

With interest in development and application of composite cereal porridges that include micronutrient rich plant materials, it becomes critical to assess the potential for interactions that can modify the bioavailability of provitamin A carotenoids. Carotenoid bioavailability depends on multiple factors including their food matrix (206,207), food preparation and processing methods (198) as well as the presence of other nutrients or factors such as lipids (208,209), fibers (113,210,211) and minerals (78,212,213) that may impact the ability of carotenoids to be released from the food matrix during digestion and associate with lipid micelles for transport into the epithelia. Corte-Real et al. (2016) demonstrated that bioaccessibility of pure carotenoids was significantly reduced with the addition of calcium, magnesium and zinc with calcium having the highest impact in limiting micellarization (up to 100% reduction). The same group later investigated the impact of divalent minerals on carotenoid bioaccessibility from carotenoids in their natural matrices and reported that while the response to the divalent minerals was dependent on the food matrices, the bioaccessibility of carotenoids was significantly reduced in the presence of the divalent minerals (78). Consequently, while food-based strategies that promote the simultaneous incorporation of vitamin A and mineral rich food ingredients are of great interest,

the impact of this complex mixture on the bioavailability of provitamin A carotenoids needs to be established.

The present study investigated the impact of incorporating baobab as a natural iron fortificant, on the bioaccessibility of provitamin A carotenoids from dried carrot and mango blend co-formulated in frequently consumed millet porridges. Considering previous reports showing the negative impact of high mineral content on the formation of mixed micelles thereby reducing the bioaccessibility of carotenoids, we hypothesized that the addition of mineral rich baobab would negatively impact the bioaccessibility of provitamin A carotenoids from a blended millet-fruit/vegetable thin porridge.

3.2 Materials and Methods

3.2.1 Materials

Carotenoid authentic standards including β-carotene, β-cryptoxanthin, as well as β-apo-8′-carotenal (internal standard) were purchased from Sigma (St. Louis, MO). α-cryptoxanthin, and α-carotene were from CaroteNature (Lupsingen, Switzerland). HPLC and ACS grade solvents petroleum ether, acetone, hexane, ethyl acetate, and methanol were purchased from J. T. Baker (Phillipsburg, NJ) and methyl tert-butyl ether was from Sigma-Aldrich (St. Louis, MO). Ammonium acetate, butylated hydroxytoluene (BHT) and potassium hydroxide were from Sigma-Aldrich (St. Louis, MO). In *vitro* digestive enzymes including α-amylase, porcine pepsin, pancreatin, lipase, and bile extract were obtained from Sigma-Aldrich. For cell culture experiments, Dulbecco's Modified Eagles Medium (DMEM), non-essential amino acids (NEAA), penicillin/streptomycin (pen/strep), and phosphate buffered saline (PBS) were purchased from Lonza (Walkersville, MD, USA). Fetal Bovine Serum was purchased from Atlanta Biologicals (Atlanta, GA). 1% v/v solution of 4-(2-hydroxylethyl)-1-piperazineethanes (HEPES) was prepared using double-distilled water, adjusted to 7.2 pH and autoclaved. Gentamycin from J.R. Scientific (Woodland, CA, USA), and trypsin from Thermo Scientific (Waltham, MA, USA) were obtained. bovine serum albumin (free fatty acid free) (FFA), sodium taurocholate and QuantiPro BCA Assay Kit were obtained from Sigma-Aldrich (St.Louis, MO). Whole grain pearl millet (P. typhoides) was received from Alif Group in Darkar, Senegal which was later decorticated and extruded at

Purdue University. Fresh carrots and mangoes were purchased from local market. Dried baobab fruit powder samples were received from Free Works Production (Dec. 2016, Dakar, Senegal).

3.2.2 Preparation of blended millet-based porridge

Test materials for composite porridges consisted of traditional cereal (pearl millet) and dried carrot and mango (provitamin A sources) which would be available seasonally and locally in Senegal. The provitamin A sources added at 6% of the final porridge (w/w) provided ~103 µg retinol activity equivalent (RAE) based on the USDA Food Composition database which met ~30% of US RDA for children aged 1-3 yr. Addition of dried baobab fruit was based on the natural iron and vitamin C content. Baobab added at 5, 15 and 25% will provide ~0.1, 0.4 and 0.6 mg of iron respectively which, along with the pearl millet (~1 mg of iron), is predicted to meet 15-23% of RDA for 1-3yr old children. Prior to test porridge preparation, the millet was decorticated and fully cooked by extrusion using a Technochem Mini-Extruder© (speed fixed at 900 rpm; final temperatures ranging between 105-121 °C). The extruded millet powder was sieved (mesh size openings of 180 to 300 µm) to control for particle size differences. Carrots and ripe mangos were trimmed and sliced into 1mm diameter using Professional Grade Quality Kitchen mandoline, and freeze-dried (Labconco Freezone 18 L, Kansas, MO, USA) and ground in a blender (Cuisinart Spice and Nut Grinder, East Windsor NJ, USA) to generate a powdered ingredient. Preparation of final test porridges followed formulas described in Table 1. These products were designed to emulate thin porridges commonly consumed in Senegal and prepared as described by Lipkie et al. (2013) with modifications (120). Briefly, individual ingredients were directly weighed into a falcon tube for a total of ~1.9 g of dry mix. The porridge slurry was then prepared by adding 8 ml of distilled boiling water (1:4, dry mix to water ratio), agitated on a vortex mixer for 5 min for thorough mixing, and placed under room temperature for 30 min before storage at -80C or further analysis or digestion

3.2.3 Carotenoid bioaccessibility from blended cereal porridge samples

Prior to digestions, porridges were thawed and brought to room temperature. To facilitate the incorporation of carotenoids into mixed micelles, ~0.1g (1% w/w) sun flower oil was added to the 9.9g of prepared porridge for a total of 10 g prior to initiation of in vitro digestion experiment. Each 10 g porridge sample was then subjected to a three-stage simulated digestion consisting of

an oral, gastric and small intestinal phase as previously described with minor modifications (120). Briefly, the oral phase solution (6 mL) containing α- amylase (3000 units) was added into the porridge sample. The reaction tube was then thoroughly mixed on a vortex for 1 min, flushed with nitrogen and incubated for 10 min in a shaking incubator at 120 rpm. Subsequently, samples were subjected to a gastric phase which consisted of the addition of 2 mL pepsin solution in 0.1M HCl (10 mg/mL) and pH adjustment to 2.5 using 1M HCl. The reaction tube volume was then brought to 40 mL with 0.9% saline solution, flushed with nitrogen and incubated (120 rpm) at 37 °C for 1hr. Following the intestinal digestion, aliquots of chyme were collected and stored at -80C while a separate aliquot of the same digesta sample was centrifuged at 10,000g at 4 °C for 1 hr to isolate carotenoids sequestered in mixed micelles within the aqueous fraction from solid residues and other insoluble particles. The aqueous fraction was then filtered with 0.22 μm PTFE to isolate the mixed micelle fraction which was collected and stored at -80 °C until further analysis.

3.2.4 Caco-2 cell culture and cellular uptake experiment

Following in vitro digestion, carotenoid uptake from aqueous digesta fractions by the human intestinal cells was evaluated using highly differentiated human intestinal Caco-2 cells (HTB-37, American Type Culture Collection). Cells were maintained as previously described with minor modifications (214). Caco-2 cells were grown at 37 °C with 5% CO_2 atmosphere and passaged ~80-85% confluency. Cells were maintained using complete media composed of DMEM containing 10% v/v FBS, 1% v/v NEAA, 1% v/v HEPES, 1% v/v pen/strep and 0.1% v/v gentamicin where the media was changed every 2 days and 24 hours before uptake experiment. Cells at passages 23-29 were then grown on 6-well plates at a seeding density of 1.28 x 10^5 cells/well and were used for uptake experiments 10-12 days post confluency. To evaluate carotenoid uptake and accumulation by the Caco-2 cells, the differentiated cells were rinsed with 0.1% fatty acid free albumin in PBS at 37 °C followed by PBS at 37 °C alone. The cells were then incubated with test media containing (1:4) aqueous digesta and DMEM for 0, 2, 4 or 6hr. Following treatment, monolayers were washed with 5mM sodium taurocholate in PBS at 37 °C post incubation to remove residues of mixed micelles and surface carotenoids and harvested in ice cold PBS (214). Protein content of treatment wells were evaluated using QuantiPro BCA Assay. Cytotoxicity of treatment media was evaluated using MTT assay.

A second experiment was also conducted to isolate the potential effects of baobab alone on carotenoid intestinal uptake without a digested porridge background. For this experiment 0.5g of baobab (to match baobab content of 25% baobab porridge sample), 1g of fruit blend (provitamin A rich carrot: mango, 1:1) and 0.9% saline sample (blank) were separately subjected to in vitro digestion and aqueous fractions from each were blended in a ratio of 1:0:1 (control), 1:1:1 (baobab_1x) or 1:2:0 (baobab_2x) respectively for the preparation of treatment media. Each of these combinations of aqueous fraction was then diluted with serum-free DMEM in a ratio of 1:3. Caco-2 monolayers were incubated with treatment media for 0, 3 and 6hr. Collected cells were stored under N2 at – 80 °C until analysis.

3.2.5 Carotenoid extraction and analysis

Carotenoid analysis from porridge samples as well as fractions collected from the digestion process were carried out according to the procedure described by Tristan et al. (120). Briefly, prepared porridge samples (0.5 g) were extracted with acetone followed by methyl tert-butyl ether (MTBE) containing 0.1% butylated hydroxytoluene (BHT). Digested porridge fractions including the intestinal digesta and aqueous micellar fractions were extracted using 0.1% BHT acetone:petroleum ether (1:3). Carotenoids from harvested cells were extracted in similar fashion as digested fractions once cell pellets were homogenized with a Branson 450 for 5 s at 20 watts . Carotenoids were analyzed using Waters ACQUITY UPLC equipped with diode array detector. Separation of carotenoids was carried out using YMC C30 3μm, 2.0 mm × 150 mm column with a gradient of ethyl acetate and methanol and 2mM ammonium acetate as previously described (119). Identification and quantification was carried out using Waters Masslynx software at 450 nm based on calibration curves of authentic standards for all-*trans*-carotenoids whereas *cis*-isomers were identified using previously reported UV-Vis absorption spectra and retention times and quantified based on all-*trans* response as previously reported (215).

3.2.6 Mineral and proximate analysis of baobab fruit powder

Dried baobab fruit powder (~ 0.5 g) was dissolved in 70% NHO_3 and digested in a closed-vessel microwave oven. Following digestion and subsequent cooling, ashed baobab powder was diluted with double-distilled water to a final concentration of 2% NHO_3 prior to mineral analysis. Analysis of calcium, magnesium, sodium, iron and zinc was carried out using inductively coupled

plasma-optical emission spectrometry (ICP-OES; Optima 4300DV, Perkin Elmer, Shelton, CT, USA). Proximate analysis including crude protein, nitrogen, crude fat, ash, crude fiber, and carbohydrates for dried baobab fruit powder was conducted by A&L Great Lakes Laboratories (Fort Wayne, IN).

3.2.7 Data analysis

Data for carotenoid content of porridge samples, digesta and aqueous fraction as well as intracellular content are expressed as mean ± SEM for a minimum three replicates for each experiment. Micellarization efficiency (relative bioaccessibility) is expressed as the ratio of the carotenoid content in the aqueous fraction to that of the digesta fraction of the porridge samples. Bioaccessible content (µg/100g, FW) is defined as the fraction of carotenoids in the starting material (porridge, µg/100g, FW) that is available for absorption as derived from the micellarization efficiency. Total provitamin A carotenoid content (TPVA) was calculated as the sum of all-*trans*-β-carotene + 1/2(α-cryptoxanthin + β-cryptoxanthin + α-carotene + *cis*-β-carotene) Carotenoid uptake efficiency (%) by Caco-2 cells was expressed as the ratio of Caco-2 carotenoid intracellular content to that of the aqueous fraction content within treatment media. Significant differences ($p < 0.05$) in individual carotenoid species and TPVA content between porridge formulations, micellarization efficiency data, bioaccessible content, cellular carotenoid content and cellular uptake efficiency were compared by analysis of variance (ANOVA) using JMP version 12 (SAS Institute, Cary, NC) with Tukey-Kramer Honestly Significant Difference (HSD) method post-hoc test.

3.3 Result and discussion

3.3.1 Provitamin A carotenoid composition of porridge meals

Freeze-dried carrots and mangoes incorporated into porridge formulation were the only sources of carotenoids as the other ingredients (millet and baobab) did not contain detectable amounts of carotenoids (data not shown). The major provitamin A carotenoids in the porridge test meals were all-*trans*-β-carotene, α-carotene, and β-cryptoxanthin consistent with previous reports of fresh and processed carrots and mangoes (19–12). Isomers of β-carotene including 15-*cis*-β-carotene, 13-*cis*-β-carotene and 9-*cis*-β-carotene were also detected and presumably derived from

the drying/preparation process as well as some level of natural *cis* isomer content (216). TPVA content of prepared porridge meals ranged from 3,666.9 ± 45.3 to 3,694.1 ± 26.9 μg/100g of fresh weight with no significant differences detected between porridge formulations ($p > 0.05$) (**Table 3.1).** Consistent with previous reports on carrots and mango, all-*trans*-β-carotene was the predominant provitamin A carotenoid contributing ~74% of the total provitamin A carotenoid concentration (29,30). α-carotene contributed ~23% taking into account that α-carotene has been reported to have 50% the provitamin A activity as β-carotene (219). β-cryptoxanthin concentration was the lowest among all the carotenoid species identified contributing only ~0.24% of the total provitamin A carotenoid concentration. Total *cis*-isomers accounted for only ~2.9% of total provitamin A carotenoids which is consistent with previous reports showing the presence of β-carotene isomers in fresh and processed carrots (220,221).

Table 3-1 Formulation and carotenoid content of porridge meals[1,2,3]

	Control	Baobab at 5%	Baobab at 15%	Baobab at 25%
Ingredients (% wt./wt.)				
Water	80.0	80.0	80.0	80.0
Decorated & extruded Souna millet	13	12	10	8
Carrot & mango blend (1:1)	6	6	6	6
Baobab	0	1	3	5
Sunflower oil (5% of the dry mix)	1	1	1	1
Total wet porridge (dry mix + water) %	100	100	100	100
Carotenoid content (μg/100g fresh wt)				
β-cryp	18.3 ± 0.9	17.8 ± 0.8	17.1 ± 1.0	15.7 ± 0.6
All-trans-αC	1728.1 ± 13.3	1719.2 ± 27.8	1679.3 ± 4.6	1715.8 ± 19.3
All-trans-βC	2719.5 ± 23.3	2707.8 ± 40.5	2635.9 ± 22.5	2694.5 ± 34.4
cis-βC	211.6 ± 7.0	200.9 ± 10.8	213.2 ± 5.4	219.3 ± 7.0
Total Provitamin A carotenoids (TPVA)	3698.5 ± 26.5	3676.7 ± 58.2	3590.7 ± 23.4	3669.9 ± 45.3

[1]Reported values represent mean ± standard error of the mean of n=4 analysis.
[2]Abbreviations used: β-cryptoxanthin (β-CRP), all-*trans*-α-carotene (all-trans-αC), all-*trans*-β-carotene (all-trans-βC), *cis*-β-carotene isomers (cis-βC).
[3]TPVA represents total provitamin A carotenoids calculated as β-carotene equivalent calculated as follows: all-trans-βC + 1/2(β-cryp + all-trans-αC + cis-βC). No significant differences ($p > 0.05$) as analyzed by Tukey's test were observed between porridge meals within individual carotenoid species.

3.3.2 Bioaccessibility of provitamin A carotenoids from porridge meals

The main objective of this study was to determine the potential effects of formulating blended millet porridges with natural sources of provitamin A (carrot and mango) and iron (baobab)

on the bioaccessibility of provitamin A carotenoids. Using a three-stage in *vitro* digestion model, micerallization efficiency (relatively bioaccessibility, %) from formulas matched for provitamin A content but increasing proportions of baobab were assessed. Overall, micellarization efficiency was found to be negatively impacted by presence of baobab in a dose-dependent fashion (**Figure 3.1**). Micellarization of TPVA ranged from 13.4% (baobab at 25%), 18.58% (baobab at 15%), 20.46% (baobab at 5%), to 23.5 ± 1 (control). While there were no significant differences in total provitamin A carotenoid micellarization between lower baobab addition (baobab at 5 and 15%) into porridge formulations as compared to the control, the presence of baobab at 25% significantly reduced ($p < 0.05$) total provitamin A carotenoid bioaccessibility.

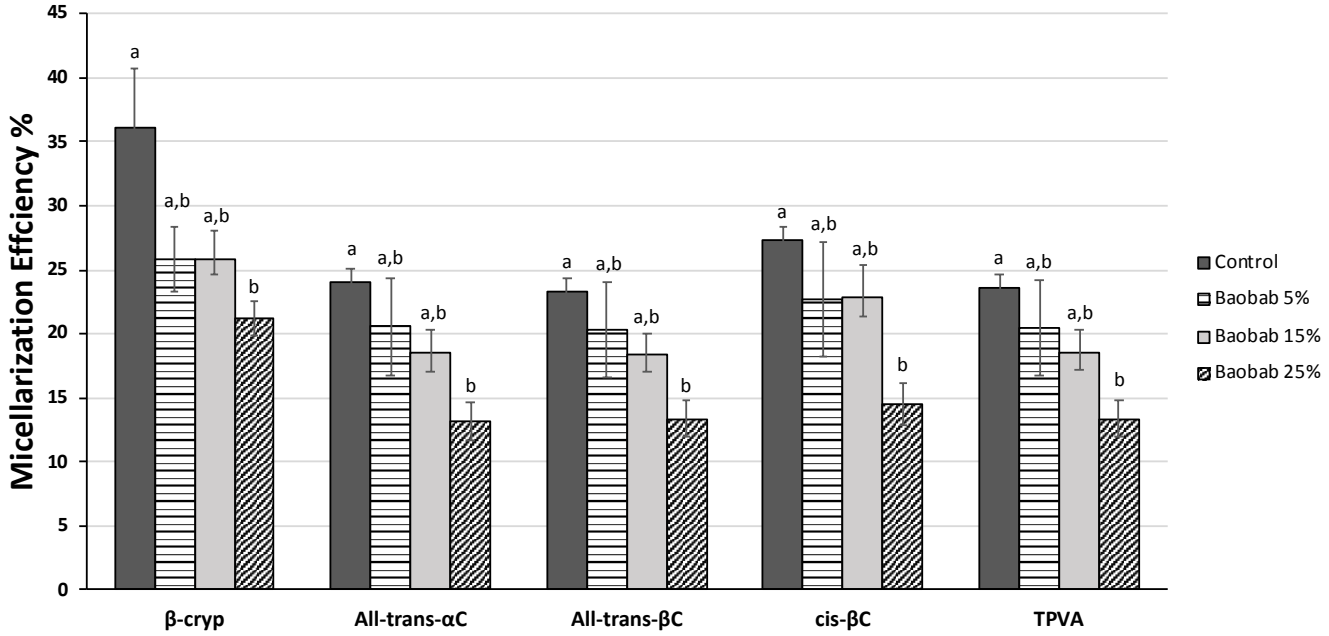

Figure 3.1 Percent micellarization efficiency (relative bioaccessibility) of individual carotenoid species from control, baobab at 5%, baobab at 15%, baobab at 25% digested porridge meals. All data presented are expressed as mean ± standard error of the mean of n=4 analyses. Different letters indicated above the bars represent significant differences in micellarization efficiency (%) between porridge formulations within individual carotenoid species ($p < 0.05$). [Grab your reader's attention with a great quote from the document or use this space to emphasize a key point. To place this text box anywhere on the page, just drag it.]

Reduction of micellarization efficiency may be due to several factors including the contribution of divalent minerals from baobab that may interfere with the formation of mixed micelles. Minerals analysis of the baobab utilized in these studies found high levels of calcium (2922.6 µg/g) and magnesium (and 1501.1 µg/g) (**Table 3.2**). A study by Crote-Real et al. (2017) showed that the micellarization efficiency of β-carotene from carrots was significantly reduced with increased levels of calcium (0 -1000 mg/l) and magnesium (0 – 300 mg/L) (78). The authors demonstrated that increased levels of divalent minerals resulted in higher surface tension and macro-viscosity indicating the loss of surfactants (bile salts) from the micellar structure. The divalent metals may also cause precipitation of insoluble fatty acid soaps as well as bile salts by binding to unconjugated fatty acids and bile acids (78,114,211). These factors likely lead to disruption of mixed micelle formation and/or stability resulting in and overall reduction of carotenoid bioaccessibility.

Table 3-2 Nutritive and non-nutritive components of baobab fruit 1

Proximate analysis (%)						
Moisture	**Nitrogen**	**Crude Protein**	**Crude Fiber**	**Crude Fat**	**Ash**	**Carbohydrates**
7.1± 0.1	0.4± 0.0	2.5± 0.1	11.2± 0.4	0.5± 0.0	4.5± 0.0	74.3± 0.4

Mineral content (µg/g)						**Total phenols (µg/g)**
Calcium	**Magnesium**	**Sodium**	**Phosphorus**	**Iron**	**Zinc**	**Gallic acid eq.**
2922.6 ± 229.1	1501.1 ± 104.6	ND	500.3 ± 68.9	118.3 ± 19.9	ND	1211.4 ± 187.0

[1]Reported values represent mean ± standard error of the mean of n=3 analysis

Additional potential factors limiting the micellarization of carotenoids could be the presence of specific phenolics (i.e. flavan-3-ols) and polysaccharides (xyloglucans) in baobab fruit as described in **Chapter 2**. Flavan-3-ols, particularly procyanidins have been shown to limit pancreatic lipase activity in mice and in human known to contribute to their role in management

of obesity and diabetes (175). As the major phenolics found in baobab were found to be flavan-3-ols, the limited micellarization of carotenoids may be partially attributed to this inhibitory effect of lipase activity by these classes of compounds. In terms of polysaccharides, while the effect xyloglucan (identified as soluble fraction of fiber) on the bioaccessibility and bioavailability has not been investigated, other soluble and insoluble fibers have been shown to limit carotenoid bioavailability by precipitating and increasing excretion of bile salts (113,222). It could be predicted that xyloglucans from baobab might have similar inhibitory effects although further *in vitro* and *in vivo* studies are required demonstrating the binding effect of xyloglucan isolated from baobab on bile salts and other micellar components to establish their effect on carotenoid bioavailability.

As indicated in **Figure 3.1**, similar negative effects were observed on the micellarization efficiency of individual carotenoid species with the addition of 25% baobab. All carotenoid species had a significantly lower micellarization ($p < 0.05$) in porridge containing 25% baobab compared to control. However, significance of this effect was not observed at lower levels of 15 and 5%. Interestingly, β-cryptoxanthin from the control (36%) and baobab at 25% (21%) porridge meals had a significantly greater micellarization efficiency as compared to α- and β-carotene. This finding is in agreement with previous reports showing higher bioaccessibility of β-cryptoxanthin as compared to α- and β-carotene (62,223). β-carotene *cis* isomers similarly demonstrated higher micellarization than all-*trans* isomer, consistent with previous observations on the micellarization efficiency of cis-isomers using a similar model (214,224). However, despite the higher relative bioaccessibility of the *cis* isomers, all-*trans*-β-carotene was the major carotenoid present in the bioaccessible fraction contributing ~72% of the total provitamin A carotenoids followed by α-carotene (~24%).

3.3.3 Bioaccessible content from porridge samples

Bioaccessible carotenoid content reflects the amount of carotenoids available from a serving of raw material adjusted by the micellarization efficiency (raw material content x %micerallization efficiency). The bioaccessible content of the porridge samples from a modest but reasonable serving size (200 g wet wt.) is presented in **Table 3.3**. Despite the similarities in TPVA content of all undigested porridge meals, the bioaccessible content was significantly lower ($p < 0.05$) from formulation containing baobab at 25% (982.6 µg per serving) than that of the control (1743.4 µg per serving) but not from baobab at 5 (1506 µg per serving) or baobab at 15% (1334.5 µg per serving). While porridge formulation containing baobab at 25% had significantly lower amounts bioaccessible α-carotene, β-carotene and cis isomers than that of the control, there no significant differences between control and baobab at 5 and 15% for these carotenoid species. However, the bioaccessible content of β-cryptoxanthin was significantly higher from control porridge (13.2 µg per serving) than all the other formulations (6.6 – 9.1 µg per serving). Overall, considering the high correlation ($r = 98$, $p < 0.05$) of carotenoid bioavailability from this in vitro model to that of healthy human studies (68), the contribution of these porridge formulations to the RDA of vitamin A can be estimated. The consumption of 200 g of porridge meals containing 0 (control), 0.2, 0.8 or 1.2 mg of iron from baobab designed to meet 30-46% of the RDA (adjusted for serving size) for iron has the potential to meet 48%, 41%, 37% or 27% of the RDA of vitamin A for children 1-3 years of age. These data indicate that the bioaccessible carotenoid content of porridge meals is reduced with high levels of baobab inclusion highlighting the potential limiting effects of mineral rich plants on the incorporation of carotenoids into mixed micelles prior to absorption by the small intestine.

Table 3-3 Absolute bioaccessibility of provitamin A carotenoids from porridge meals (µg/per serving)[1,2]

	Porridge Formulations			
Carotenoids	**Control**	**Baobab 5%**	**Baobab 15%**	**Baobab 25%**
β-cryp	13.2 ± 1.6a	9.1 ± 0.6b	8.9 ± 0.8b	6.6 ± 0.2b
All-trans-αC	830.6 ± 32.9a	707.7 ± 128.5ab	623.3 ± 56.5ab	453 ± 50.5b
All-trans-βC	1263.7 ± 52.7a	1102.1 ± 206.7ab	970.2 ± 86.6ab	720.8 ± 78.9b
cis-βC	115.6 ± 7.4a	90.6 ± 16.1ab	98.2 ± 12.2ab	63.4 ± 6.1b
TPVA	1743.4 ± 72.6a	1506 ± 279.1ab	1334.5 ± 120.7ab	982.6 ± 106.7b

[1]Data shown represent absolute bioaccessibility calculated as the (raw material carotenoid content, µg/100g fresh weight) x (micellarization efficiency, %).
[2]Values are mean ± standard error of the mean of n=4 analysis. Different represent significant differences in absolute bioaccessibility between porridge formulations within individual carotenoid species ($p < 0.05$).

3.3.4 Provitamin A carotenoid uptake and accumulation efficiency

Carotenoid uptake/accumulation by human intestinal cells was assessed using differentiated Caco-2 (HTB37, parental cell line) human intestinal cells. This model was used to further validate the impact of baobab on the delivery of the provitamin A carotenoids from micellarized fraction of digested carrot/mango blend incorporated in millet based porridge formulations. Prior to Caco-2 uptake experiment, treatment media were prepared using micellar fractions of digested porridge samples diluted with DMEM (1:4). Caco-2 cells were then incubated with treatment media from 0 to 6 hours. Among the provitamin A carotenoids detected in the micellar fraction, all-*trans*-β-carotene and α-carotene were the only provitamin A carotenoids identified in harvested Caco-2 monolayers as the intracellular content of β-cryptoxanthin and *cis* isomers were below the limit of detection.

Table 3-4 Levels of α-carotene and β-carotene present in treatment media (pmol/ml) 1,2,3

	Porridge formulations			
Carotenoids	**Control**	**Baobab 5%**	**Baobab 15%**	**Baobab 25%**
β-cryp	6.4 ± 0.7a	5.2 ± 0.2ab	3.9 ± 0.3bc	2.8 ± 0.1c
All-trans-αC	377.0 ± 13.8a	335.2 ± 27.5a	222.6 ± 17.9b	123.5 ± 3.5c
All-trans-βC	568.8 ± 18.7a	515.3 ± 41.7a	347.2 ± 27.0b	198.2 ± 5.9c

[1]Aqueous fractions collected during in vitro digestion from each porridge meals were diluted with DMEM (1:4) and used as treatment media for the subsequent Caco-2 uptake experiment.
[2]Data shown represent mean ± standard error of the mean of n=4 analysis.
[3]Different letters indicate significant differences in between porridge formulations within individual carotenoid species ($p < 0.05$).

The content of micellarized all-*trans*-β-carotene and α-carotene from test porridge samples in treatment media are presented in **Table 3.4**. Treatment media from control (568.8 ± 18.7 pmol/well) and baobab at 5% (515.3 ± 41.7 pmol/well) porridge meals had significantly greater ($p < 0.05$) all-*trans*-β-carotene content than baobab at 15% (347.2 ± 2 pmol/well) and baobab at 25% (198.2 ± 5.9 pmol/well). Similarly, α-carotene concentration was significantly greater in control (377.0 ± 13.8 pmol/well) and baobab at 5% (335.2 ± 27.5 pmol/well) than in baobab at 15% or 25% (222.6 ± 17.9 and 123.5 ± 3.5 pmol/well respectively). The uptake efficiency of both α- and β-carotene increased with increased incubation time for all porridge formulations reaching a stable level at 4hr (**Table 3.5**). No significant differences in uptake efficiency were observed between 4 and 6hr incubation period amongst all porridge formulations suggesting that absorption might be time dependent and saturable as previously documented for Caco-2 (225). By the end of the final incubation period (6hr), β-carotene accumulation by Caco-2 cells ranged from 3.7% (control), 3.7% (baobab at 5%), 3.8% (baobab at 15%) to 4.45% (baobab at 25%). Similar trends were observed for α-carotene where accumulation efficiency ranged from 3.3% (control) to 3.61% (baobab at 25%). Despite the significant decrease in micellarization efficiency of α- and β-carotene with the addition of baobab at 25% within the porridge formulations, there were no significant differences

($p > 0.05$) in the accumulation efficiency of these carotenoids between the different porridge formulations within each time point.

Table 3-5 Caco-2 uptake efficiency (%) of α- and β-carotene after 0, 2, 4 and 6hr of incubation with diluted micellar aqueous fraction from porridge meals containing 0, 5, 15 or 25% baobab 1

	All-trans-αC				all-trans-βC			
Time	**control**	**Baobab 5%**	**Baobab 15%**	**Baobab 25%**	**control**	**Baobab 5%**	**Baobab 15%**	**Baobab 25%**
0hr	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0.2 ± 0.1	0.2 ± 0.03	0.2 ± 0.1	0.1 ± 0.2
2hr	1.2 ± 0.4	0.9 ± 0.2	0.9 ± 0.3	1.3 ± 0.6	1.3 ± 0.3	1.1 ± 0.3	1.3 ± 0.3	2.0 ± 0.6
4hr	2.2 ± 0.3	1.9 ± 0.3	2.0 ± 0.3	2.6 ± 0.7	2.5 ± 0.4	2.2 ± 0.4	2.4 ± 0.3	3.3 ± 0.8
6hr	3.3 ± 0.7	3.3 ±0.5	3.3 ± 0.2	3.6 ± 0.5	3.7 ± 0.8	3.7 ± 0.6	3.8 ± 0.3	4.5 ± 0.7

Uptake efficiency by Caco-2 monolayers of all-trans-αC and all-trans-βC from control, baobab at 5%, 15% and 25% porridge meals after 0, 2, 4 and 6hr.
[1]Each data point represents mean ± standard error of percent ratio of total cellular carotenoid content to that of the aqueous fraction (n=4 wells at each time point). No significant differences were observed ($p > 0.05$).

To specifically identify any effect of baobab on carotenoid absorption by Caco-2 cells and to match the carotenoid content of treatment media, a second experiment was conducted without millet. Briefly, micellar fractions of digested fruit blend (carrot: mango, 1:1) were incubated with separately digested baobab (0.5g to match the content of baobab at 25% in porridge meals) for 0, 3 and 6hr. To match the carotenoid content of all treatment samples, digested blank sample (saline, 0.9%) was introduced and the treatments were prepared in ratios described in section 2.4.1. The treatment combinations were then diluted with DMEM (1:3) to ensure detectable amount of carotenoids is supplied to the cells for absorptions. α- and β-carotene accumulation increased linearly with increased incubation time. With control treatment, cellular α-carotene content ranged from 0 (0hr), 3.01 (3hr) to 5.9 (6hr) pmol/mg of protein. The presence of baobab did not affect the accumulation of α-carotene. Similarly, there were no significant difference in β-carotene accumulation between the different treatments.

Table 3-6 Uptake efficiency (%) of Caco-2 cells exposed to treatment media containing control, baobab_1x or baobab_2x samples incubated for 0, 3 or 6hr [1,2]

	All-trans-αC			all-trans-βC		
Time	**control**	**Baobab_1x**	**Baobab_2x**	**control**	**Baobab_1x**	**Baobab_2x**
0hr	0 ± 0	0 ± 0	0 ± 0	1.0 ± 0.3	1.3 ± 0.1	1.1 ± 0.1
3hr	3.4 ± 0.2	4.7 ± 0.3	4.6 ± 0.4	5.1 ± 0.3	7.0 ± 0.4	7.3 ± 0.6
6hr	7.4 ± 1.4	7.8 ± 0.3	7.5 ± 0.4	9.6 ± 1.3	10.7 ± 0.4	10.6 ± 0.4

[1]α-carotene and β-carotene uptake efficiency by Caco-2 cell monolayers incubated with control, baobab_1x, baobab_2x treatment samples for 0, 3, and 6hr. The description of treatments is provided in **section 3.2.4**.
[2]Each data point represents mean ± standard error of percent ratio of total cellular carotenoid content to that of the aqueous fraction (n=4 wells at each time point). No significant differences were observed ($p > 0.05$)

Cellular content of β-carotene ranged from 1.29 (control, 0hr) - 11.14 (control, 6hr) pmol/mg of protein. The uptake efficiency of Caco-2 cells incubated with the different treatment media over 0, 3 and 6hr is presented in **Table 3.6**. By the end of the incubation period (6hr), there were no significant differences in accumulation efficiency of both α-carotene (7.4 - 7.8%) or β-carotene (9.6 – 10.7%) between the different treatment groups. The uptake efficiency from the current study is consistent with previous report by Thakkar et al. (2007) who reported the percentage of medium β-carotene accumulated by differentiated Caco-2 monolayers from different cultivars of cassava to be 10-11% (226). Sullivan et al. (2007) also investigated the absorption and transport of α- and β-carotene at varying levels using Caco-2 cells and demonstrated that the uptake efficiency ranged from 5% (0.5 μg) to 4% (2.5 μg) for β-carotene and 6% (0.5 μg) to 4% (2.5 μg) for α-carotene. In contrast, other studies have reported higher levels of uptake efficiency of these carotenoids ranging from 20-40% (227,228). These differences may be due differences in dosage, form of carotenoid source (free vs. complexed in their matrices), or differences in experimental conditions for Caco-2 cell culture. Overall, the current findings are generally in agreement with in vitro studies mentioned above as well as clinical studies demonstrating similar absorption efficiency from pure β-carotene (~4%) using a double tracer study design and β-

carotene from processed carrot samples (~2%) using extrinsic stable isotope method which validate the use of Caco-2 cell models to predict carotenoid absorption by the enterocytes of the small intestine (229,230).

3.4 Conclusion

Findings from the current study indicate that increased addition of baobab in porridge formulations containing provitamin A carotenoid-rich fruit blends seemed to have an inverse relationship with carotenoid incorporation into mixed micelles prior to their absorption by the small intestine. While it is highly likely that the presence of high concentrations of divalent minerals in baobab may cause the decrease in micellarization efficiency as previously demonstrated (114,213), further research is warranted investigating the impact of baobab on the physiochemical parameters of micelles formed during the digestion process in order to establish direct relationship. However, despite the reduced bioaccessibility observed with increased amount of baobab, the absorption of target provitamin A carotenoids by Caco-2 human intestinal cells was not significantly altered in an acute setting. It remains to be seen if long-term exposure to baobab, as would be expected form introduction into staple foods, may alter lipid/carotenoid absorption by modulating expression of genes involved in carotenoid transport CD36 and SR-B1. In general, it could be estimated that these porridge formulations (from 200 g serving size) containing 5-25% of baobab designed to meet 30-46% of the RDA for iron have the potential to meet 27-48% of the RDA of vitamin A for children 1-3 years of age. Overall, the present study provides useful insights for screening food product formulations containing native nutrient dense plant materials designed for the optimal delivery of carotenoids.

CHAPTER 4. CHRONIC EXPOSURE OF NATIVE *ADANSIONA DIGITATA* (BAOBAB) AND *MORINGA OLEIFERA* (MORINGA) MODIFY CACO-2 HUMAN INTESTINAL CELL DIFFERENTIATION AND CAROTENIOD ABSORPTION

4.1 Introduction

Globally, over 2 billion people are affected by micronutrient deficiency including vitamin A, iron and zinc (231). Efforts to address these challenges include supplementation, food fortification, and diet diversification which promote the use of native African plant materials that are rich in both vitamin A and iron (236). Nutrient-dense plant materials native to Sub-Saharan Africa are receiving significant attention in fortification and dietary diversification efforts with a focus on provitamin A carotenoids and shortfall minerals including iron and zinc (203). Among these plants, *Adansiona digitata* (baobab) and *Moringa oleifera* (moringa) are recognized as potentially rich sources of iron (both) and provitamin A carotenoids (moringa) as well as other phytochemicals (phenolics and isothiocyanates) that have been associated with their health benefits and functional properties (97,103,105,237). These plant materials also have shown promise as enhancers of iron absorption when used as ingredients in composite cereal porridges commonly consumed in the Sahelian region (11). Improvement of iron and zinc absorption has been attributed to presence of known potentiators such as ascorbic acid (baobab) and other organic acids such as malic, citric, lactic and tartaric acid (238). However, when considering inclusion of complex natural fortification approaches to traditional cereal staple foods, one must also consider potential interactions that may impact delivery and bioefficiency of other shortfall micronutrients, such as vitamin A. To date, few studies have investigated such impacts from these novel approaches.

Several factors can influence the bioavailability of provitamin A carotenoids including food matrix, type and amount of co-consumed fat (208,239), dietary fiber (113). Host-related factors such as disease state including malaria and bacterial infection (240,241) and genetic variations particularly related to carotenoid transporter genes including scavenger receptor, class B type I (SR-B1) and Cluster Determinant 36 (CD36) (242–244). In regards to matrix effects, minerals have been reported to negatively influence provitamin A carotenoid bioaccessibility and absorption (79,212). Divalent minerals in particular, including calcium and magnesium specifically, have been shown to form complexes with bile salts and precipitate fatty acids which impacts mixed micelle formation and carotenoid bioaccessibility in model systems (78,212). Intestinal uptake of carotenoids may also be negatively impacted by presence of bioaccessible minerals. Bengtsson et al. (2009) reported that the co-incubation of Caco-2 human intestinal cells with ferrous chloride (30 μM) and micellarized β-carotene resulted in a reduction of β-carotene uptake (79). However, we have recently reported that while incremental addition of mineral rich ingredients such as baobab to carotenoid rich porridges did reduce bioaccessibility, no impact was observed on the acute uptake of carotenoids by highly differentiated Caco-2 cells in culture. These contrary results could be explained by differences in experimental conditions including the use of food versus purified systems.

While potential for negative effects have been previously observed (79) and direct impacts to bioaccessibility noted (78) in experimental food systems, it is important to consider these are acute exposure in tightly controlled cellular models. Formulated staple products would in fact be repeatedly consumed resulting in long term exposure to the dietary components from these plants that might result in adaptation and perhaps modify the response to provitamin A carotenoids or elicit potential toxic responses over extended periods of consumption. As such, it is not clear if

findings from studies with acute treatment are relevant to chronic exposure as the principal goal of dietary diversification strategies are focused on increasing consumption frequencies of these nutrient dense plants to address micronutrient deficiency. It is important to assess the potential for these priority plant ingredients to affect intestinal function and also carotenoid absorption over a more long term exposure paradigm. The present study investigated the impact of chronic exposure of moringa and baobab to human intestinal cells (Caco-2) on intestinal cellular differentiation by ^{1}H NMR spectroscopy and select molecular mechanism underlying subsequent provitamin A transport from experimental porridges.

4.2 Materials and methods

4.2.1 Plant materials

Dried moringa leaf powder was obtained from Maria Productions (Feb, 2016, Dakar, Senegal) and baobab fruit powder was from Free Works Production (Feb, 2016, Dakar, Senegal). Carrots were purchased from the local market, freeze-dried (Labconco Freezone 18 L, Kansas, MO, USA) and ground in a blender (Cuisinart Spice and Nut Grinder, East Windsor NJ, USA). Samples were stored at -20 °C until further analysis.

4.2.2 Three-stage in vitro digestion

In vitro digestion model simulating the human oral, gastric and small intestine digestion was used based methods previously described (119,120). Each replicate (n=4) of digestion reaction tubes contained 0.3 g of baobab, 0.3 g of moringa, 0.5 g of carrot or 0.9% saline solution as control sample. These levels of baobab, moringa and carrot are based on the amounts incorporated in thin porridge formulations at 15% (baobab and moringa) and 30% (carrot) of dry mix respectively as shown in our previous study (Chapter 3) to have impact on carotenoid

bioaccessibility but also associated with delivery of sufficient bioaccessible iron Merwe et al. (2018). Based on our findings described in **Chapter 2** and **3**, a summary of nutritive and non-nutritive content of baobab and moringa and the expected carotenoid content of porridge formulations is presented in **Table 4.1.** 0.025 g (5% w/w) of sun flower oil commonly used in Senegal, was added to the carrot digestion to standardize lipid levels and facilitate the micellarization of carotenoids. Following the intestinal digestion, aliquots of digesta were centrifuged at 10,000g at 4 °C for 1 hr to separate the aqueous fraction from insoluble residues. Aliquots of aqueous fraction was then filtered with 0.22 μm PTFE to remove any contaminants and stored at -80 °C until further analysis.

Table 4-1 Nutritive and non-nutritive content (mg/100g, DW) of baobab fruit and moringa leaf powder, and carotenoid content of test meal 1,2,3

		mg/100g	
		Baobab	**Moringa**
minerals	Ca	292.3 ± 22.9	2325 ± 116.7
	Mg	150.1 ± 10.5	850 ± 39.7
	Na	trace	13.8 ± 0.6
	K	1963 ± 131.7	845.7 ± 40.1
	P	50 ± 6.9	229.9 ± 16.6
	Fe	11.8 ± 2	23.7 ± 0.8
	Zn	trace	0.4 ± 0.1
	Mn	0.7 ± 0.1	6.6 ± 0.4
Phenolics	Flavonols	5353.7 ± 124.9	31 ± 0.1
	Flavan-3-ols	trace	1234.6 ± 8
	Phenolic acids	276.7 ± 7.8	12.2 ± 0.7
Organic acids		982.1 ± 57.9	445.3 ± 80
Carotenoid content (µg/100g fresh, wt) of porridge formulation containing 30% carrot (dry mix)			
Carotenoids	β-cryp	17.1 ± 1.0	
	All-trans-αC	1679.3 ± 4.6	
	All-trans-βC	2635.9 ± 22.5	
	cis-βC	213.2 ± 5.4	
	TPVA	3590.7 ± 23.4	

[1]Values are mean ± standard error of triplicate analysis
[2]Abbreviations: DW, dry weight, TPVA, total provitamin A carotenoids.
[3]Carotenoid content of test meal is based on the addition of 0.5 g of carrots and 0.3 g of baobab or moringa in porridge formulations

4.2.3 Cell culture and treatments

Cell culture experiment was carried out using Caco-2 HTB37 cells (passages 25-31) obtained from American Type Culture Collection, Manassas, VA. Cells were cultured at a seeding density of 1.28 x 10^6 cells/well onto 6-well culture plates (Corning Inc., Corning, NY) and maintained using complete media containing Dulbecco's modified Eagle's medium (DMEM) with 10% v/v fetal bovine serum (FBS), 1% 1% v/v N-(2-hydroxyethyl)piperazine-N′- ethanesulfonic acid (HEPES), 1% v/v penicillin/streptomycin, 1% v/v nonessential amino acids, and 0.1%

gentamicin as previously described (245). Cells were grown at 37 °C with 5% CO_2 atmosphere and growth media was changed every 48 h with fresh media. Each cell experiment was conducted with n=4 replicate.

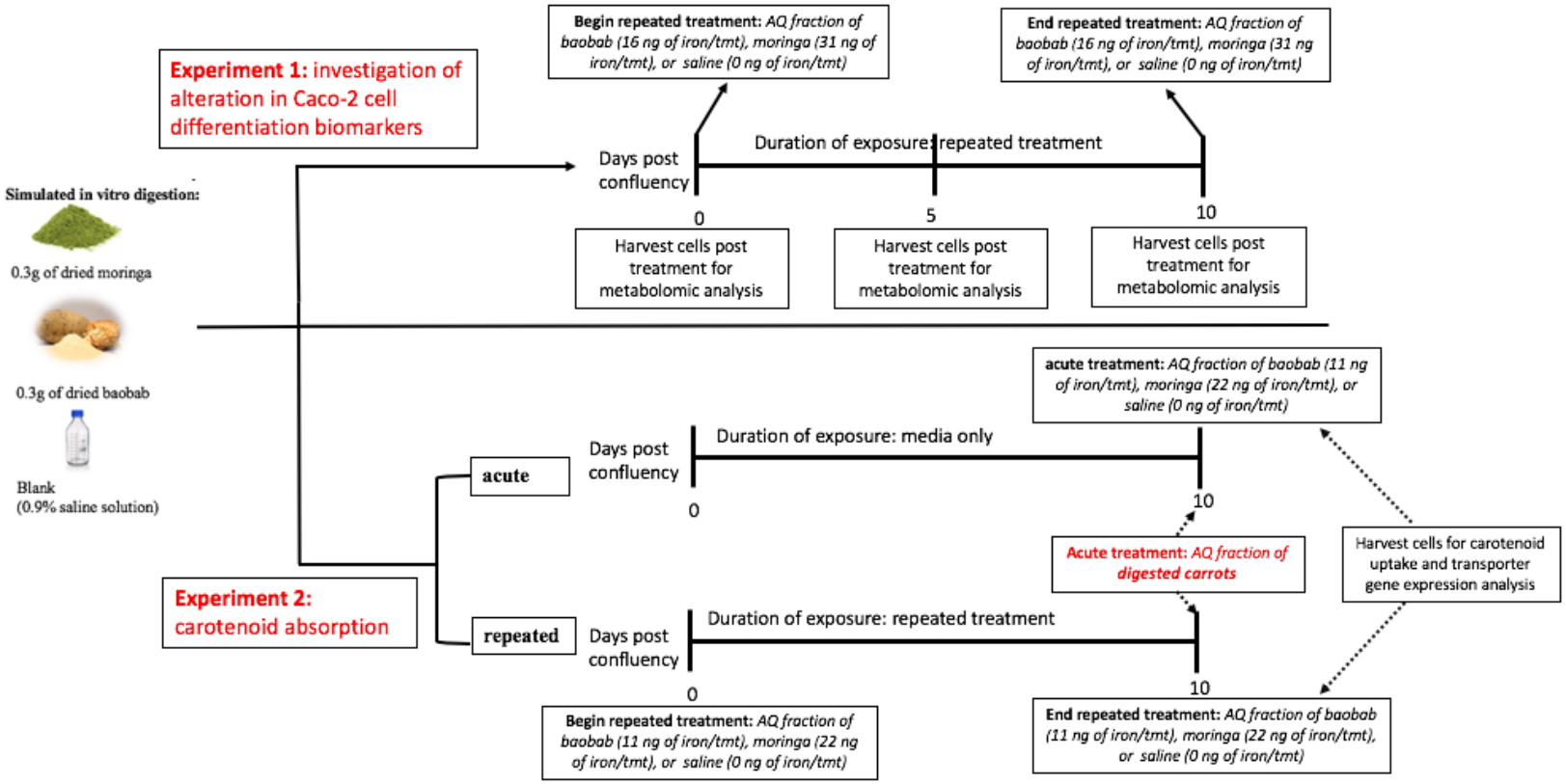

Figure 4.1 Experimental design of acute and chronic treatment of Caco-2 cell monolayers. Two separate experiments were conducted to 1) investigate the impact of chronic exposure of digested baobab and moringa on Caco-2 differentiation biomarkers and 2) investigate the impact of chronic and acute exposure of digested baobab and moringa on carotenoid absorption from acute treatment of digested provitamin A carotenoid-rich carrots.

4.1.1. Intracellular metabolite isolation for Caco-2 cell differentiation biomarkers

To investigate the impact of chronic exposure of baobab and moringa on Caco-2 cell differentiation, cells were cultured with complete media combined with aqueous fraction of digested baobab, moringa or blank saline in a 1:8 ratio (v/v) and harvested at 0, 5, 10 and 14 days post-confluency. Based on this dilution factor, incorporating baobab and moringa at 15% of staple porridge formulations is estimated to deliver 16 ng of iron/treatment (baobab) and 31 ng of iron/treatment (moringa) based on the estimated average bioaccessibility (~10%) of non-heme iron (246). Preliminary experiments were conducted to determine a range of dilution factors (1:4 – 1:12) for chronic treatment as an appropriate level inducing no significant variation in cell viability as determined by MTT assay. Treatment media was changed with fresh DMEM without aqueous fractions 24 h prior to harvesting cells for NMR experiments. Intracellular metabolite isolation at 0, 5, 10 and 14 day post confluency was conducted to follow differentiation based on methods previously described by Lee et al. (2009) with minor modifications (247). Briefly, Caco-2 cell monolayers were placed on ice to minimize metabolite degradation and harvested by first washing with 5mM sodium taurocholate at 37 °C to remove surface residues of digestion by-products, followed by thorough washing with PBS at 37 °C and finally collecting by scraping in ice cold PBS. The cell pellets were immediately snap-frozen and stored at -80 °C until further analysis. To isolate metabolites, cell pellets were thawed on ice and resuspended in 1500 µL of 80% methanol solution and vigorously mixed for every 5 min for 20 min. Then cells were homogenized using FastPrep®-24 homogenizer (MP Biomedicals, Santa Ana, CA). After that, homogenized cells were centrifuged at full speed (13,000 rpm) at 4°C for 20 min. The supernatant was collected and methanol was evaporated using a SpeedVac concentrator (Thermo Fisher Scientific, Waltham, MA).

4.2.4 NMR Metabolomic Analysis

Nuclear magnetic resonance (NMR) spectroscopy was used to identify and quantify individual targeted metabolites in cell extracts. Cell extracts were thawed, reconstituted in phosphate buffer (150 µl, 0.1M, pH=7.4) containing NaN3 (3 mM), and DSS-D6 (0.11 mM). The samples were centrifuged at 14,000 rcf for 15 min at 4 °C before transferring 590 µL into 5 mm NMR tubes (Bruker Biospin, Billerica, MA, USA). The samples were analyzed on an Avance III HD 600 MHz Bruker NMR spectrometer equipped with a Bruker SampleJet cooled to 5.6 °C. Data were acquired using a one-dimensional (1D) experiment with T2 filter using Carr–Purcell–Meiboom–Gill (CPMG) pulse sequence with water presaturation for metabolite quantitation and two-dimensional (2D) 1H-13C heteronuclear single quantum correlation (HSQC) and HSQC–TOCSY (HSQC–total correlation spectroscopy) for metabolite identification. Metabolites were identified using Bruker AssureNMR software (Bruker Biospin, USA) with BBiorefcode metabolite database and COLMARm (248). The spectra were processed using Bruker Topspin 3.6 software and in-house MATLAB scripts. All raw and processed data is available on the Metabolomics workbench (http://www.metabolomicsworkbench.org/), along with detailed experimental NMR and analysis methods.

4.2.5 Carotenoid uptake by Caco-2 cells

A parallel experiment was conducted to determine the impact of chronic and acute exposure to baobab or moringa on the intestinal uptake/accumulation of carotenoids and to understand if changes in metabolism/differentiation impacted these measures. For chronic exposure, Caco-2 cells were grown in complete media containing aqueous fraction of digested baobab (11 ng of iron/treatment) and moringa (22 ng of iron/treatment) or blank saline for 10 days post-confluency. The load of digested baobab and moringa was reduced from the metabolomic

experiment in order to accommodate the addition of digested carrots into the dilution. Treatment media was changed with fresh media containing no added aqueous fraction 24 h before uptake experiment. Carotenoid uptake experiment was carried out according to methods previously described (245). Briefly, fully differentiated Caco-2 monolayers were rinsed with 0.1% fatty acid-free albumin in PBS (37 °C) followed by PBS and incubated for 4 h with treatment media containing aqueous micellar fraction of digested carrot samples in DMEM (1:4, v/v) to match levels introduced in our previous experiments (Chapter 3). Following 4 h incubation with treatment media, cells were washed with 5mM sodium taurocholate in PBS (37 °C) to remove surface micelles and carotenoids followed by PBS (37 °C) and collected with ice cold PBS. Collected cell pellets were flushed under nitrogen and stored at -80 °C until further analysis. For acute exposure study, a parallel cell experiment was conducted in which differentiated monolayers grown with media only were acutely treated with the same amount of aqueous fraction of baobab, moringa or blank saline used in chronic exposure study. Cells were harvested and stored following 4 h incubation as described in the chronic study. Representative wells from each treatment plate were used for protein content analysis using QuantiPro BCA Assay.

4.2.6 Analysis of treatment media and intracellular carotenoid content

Carotenoid analysis from treatment media and intracellular content was carried out according to the procedure described by Tristan et al. (245). Separation and identification of carotenoids was carried out using Waters ACQUITY UPLC equipped with diode array detector equipped with YMC C30 3μm, 2.0 mm × 150 mm column with gradient system previously reported by our group (119). Authentic standards were used for identification and quantification of all-trans-carotenoids whereas cis-isomers were identified using previously reported UV-Vis absorption spectra and retention times and quantified based on all-trans response (119,249).

4.2.7 Gene expression and protein analysis

To further elucidate the impact of acute and chronic exposure to baobab and moringa on the absorption of carotenoids as well as iron, a gene expression study was carried out parallel to the carotenoid uptake experiment, with cells treated in a similar fashion. mRNA and protein levels of genes involved in the transport of carotenoids and iron from treated Caco-2 cells was evaluated using real-time reverse transcriptase-polymerase chain reaction (RT-PCR) and Western blot.

4.2.8 Quantitative RT-PCR

Total RNA was extracted using an AURUM total RNA Mini Kit with DNase digestion (Bio-Rad), according to the manufacturer's recommended procedure. First-strand cDNA was generated from 1 µg of total RNA using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR (qPCR) was performed using TaqMan® Fast Advanced Master Mix (applied biosystem, Thermo Fisher Scientific) on The LightCycler® 480 Real-Time PCR system (Roche). Gene expression level for endogenous controls was determined using pre-validated Taqman Gene Expression Assays (Applied Biosystems) as follows: FABP1 (Hs00155026), SCARB1 (Hs00969821), ABCG5 (Hs00223686), SLC11A2 (Hs00167206), CD35 (Hs00354519). qPCR reactions for 96 well plate formats were performed using 2 µl of cDNA samples (5-25ng), 10 µl of TaqMan Fast Advance MasterMix (Applied Biosystems), 1 µl of the TaqMan Gene Expression Assay (20x) and 7µl of water in a total volume of 20 µl. All reactions were performed in triplicate and the Real-time PCR was initiated under the following conditions: UNG activation step for 2 min at 50°C, Polymerase activation step for 20 sec at 95°C and then 45 cycles at 95°C for 3 sec and 60°C for 30 sec. During the reaction, fluorescence, and therefore the quantity of PCR products, was continuously monitored by Light-Cycler 480 - Real-Time PCR system (Roche). Gene expression was quantified by obtaining the number of cycles to reach a predetermined threshold

value in the intensity of the PCR signal (C_T value). Beta Actin was employed as reference gene (Assay Hs99999903) and the samples were compared using the relative cycle threshold (CT).

4.2.9 Western blot analysis

Harvested Caco-2 cells were washed with PBS and extracted in ice-cold Ripa buffer (Alfa Aesar MA, USA) combined with protease and phosphatase inhibitor cocktails (Thermo Scientific, IL, USA) for the preparation of cell lystates. Lysates were cleared by centrifugation (13,500 rpm) for 15 min at 4 °C and protein concentration was measured by Bradford method (BioRad, CA, USA). Proteins (20 μg) were separated on 10% SDS-PAGE, transferred onto nitrocellulose membrane, and blocked for 1hr in Tris-buffered saline, pH 7.5, containing 0.5% Tween 20 and 5% milk. Membranes were incubated overnight at 4 °C with the appropriate primary antibodies: FABP1 (Fitzgerald Industries International INC., MA, USA), Ferritin (Fitzgerald Industries International INC., MA, USA), SR-B1(Fitzgerald Industries International INC., MA, USA), SLC11A2 (Fitzgerald Industries International INC., MA, USA), and β-actin (Sigma, USA). The membranes were then incubated with horseradish peroxidase conjugated secondary antibody for 1hr, and the bound antibodies were detected by chemiluminescence (BioRad, USA)). β-actin was used as loading control. Images of the bands were digitized and the densitometry analysis was performed using Image J software.

4.2.10 Statistical analysis

All data are expressed as mean ± SEM of at least n=3 replicates. Statistical analysis of impact of treatment group and stage of differentiation (time) as well as the interaction between the two parameters on changes in metabolite concentrations were determined using a Two-way ANOVA consisting of treatment group, time, replicates as variables. Individual effect tests were carried out when interaction terms were not significant ($p > 0.05$). Analysis of the effect of each treatment

group on gene expression of target transporter were determined using analysis of variance (ANOVA) with post-hoc Tukey's HSD test analysis. Student's t-test was applied for those values showing significant differences with ANOVA analysis but not Tukey's HSD test. Wilks' Lambda test was for multivariate analysis of variance (MANOVA) test used to determine differences between treatment groups on all the metabolites identified. Differences were considered significant at $P < 0.05$.

4.3 Results and discussion

4.3.1 Chronic exposure of Caco-2 cells to baobab and Moringa digesta alter specific metabolic markers of cellular differentiation

The present study evaluated the biochemical alterations in Caco-2 cells associated with repeated exposure to digesta of mineral-rich baobab and moringa using metabolomic approach based on high resolution ^{1}H NMR. Caco-2 monolayers were treated from confluency and through typical differentiation period (14 days) with aqueous fractions of digested baobab, moringa or blank saline (control) and collected at specific time points during differentiation (0, 5, 10 or 14 days post-confluency) to monitor changes in targeted metabolite levels previously identified as potential biomarkers of differentiation (247). While ^{1}H NMR analysis revealed the presence of numerous distinct metabolites, substantial changes were observed only for a select number metabolites including glycerophosphocholine, taurine, myo-inositol, AMP and PNP. Similar findings were reported by Lee et al. (2009) in which they demonstrated that the levels of these metabolites, particularly taurine and myo-inositol, changed significantly during Caco-2 cell differentiation highlighting their role as potential biomarkers of differentiation (247). Multivariate discriminant analysis was carried out to better understand the overall trend and identify clustering or potential outliers of treatment groups (**Figure 4.2**). Results showed that at day 0 of post

confluency, all treatments aligned in a similar fashion with no significant differences between treatment groups as determined by Wilks' Lambda test. However, significant separation of treatments was observed at day 5 post confluency in which baobab and control treatments were clustered together and moringa treatment began to shown distinct separation. By day 10, separation of moringa from the other treatments was still observed although the differences between the treatment groups were not found to be significant.

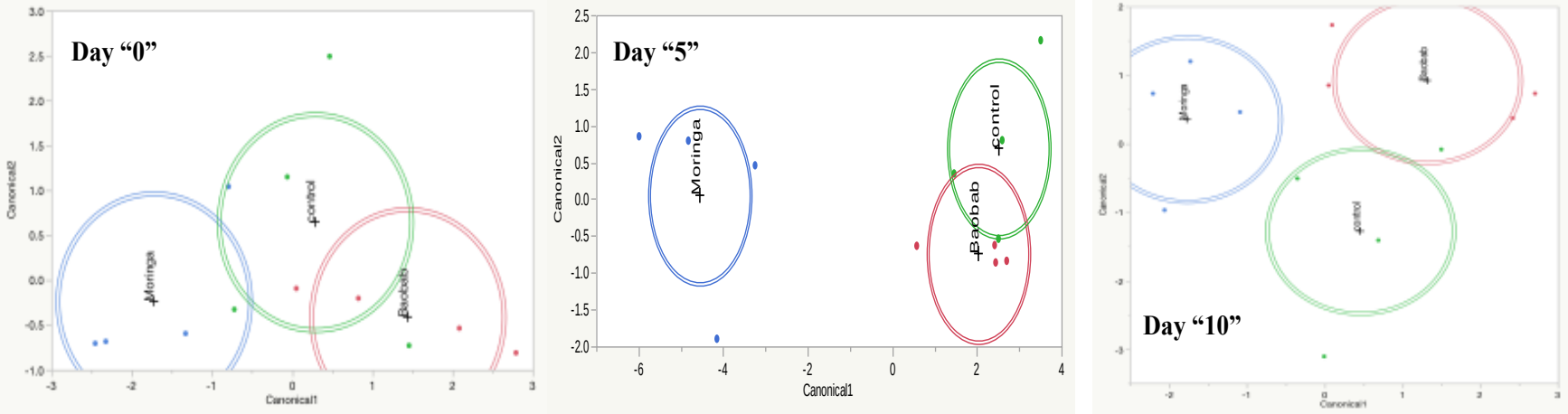

Figure 4.2 Multivariate discriminant analysis was carried out to determine the overall trend in clustering or potential outliers of treatment groups. The different clustering color groups represent each treatment (blue = moringa, green = control, red = baobab). The points represent all metabolites that showed significant changes over the differentiation period with treatment of aqueous fraction of digested baobab, moringa or control. Clusters contain 4 samples (wells) measured in triplicates. Significant clustering was observed at day 5 of differentiation as determined by Wilk's Lambda test

In the present study, glycerophosphocholine, taurine and PNP content within Caco-2 monolayers were found to increase over the course of the differentiation period for all treatment groups. Since there was no significant interaction effect between treatment groups and the different stages of differentiation (time) on the intracellular content of these metabolites, individual effect test was conducted to explore the potential impact of specific treatment type and time independently. Glycerphosophocholine content was significantly higher ($p < 0.0001$) with chronic treatment of baobab as compared to moringa treatment while there were no significant differences between control and baobab or control and moringa treatment. Furthermore, intracellular content of glycerophosphocholine was significantly lower ($p < 0.0001$) at day 0 whereas no significant differences were observed between day 5 and 10 across all treatment groups. Significantly lower glycerophosphocholine content at day 0 with all treatment groups including the control indicates that its expression in Caco-2 cells may only be initiated in fully differentiation cells. Glycerophosphocholine is produced from the consecutive removal of fatty acyl chains from its precursor, phosphatidylcholine which is the major phospholipid in the surface monolayer of intracellular lipid droplets (250). The fatty acids released from the production of glycerophosphocholine can be re-esterified into phospholipids or converted into triglycerides (251). It has been shown that reduced levels of intracellular glycerophosphocholine content due to reduced *de novo* phosphatidylcholine synthesis resulted in limited apoB48 chylomicron secretion in Caco-2 cells (252). As it has been shown that 80% of the total β-carotene secreted by Caco-2 cells was associated with chylomicrons (225), the assembly and secretion of chylomicrons is essential for the transport of carotenoids across the enterocyte and their subsequent distribution into target tissues. Consequently, maintaining intracellular glycerophosphocholine levels as seen in the current study with all treatment groups as compared to the control is critical not only for its

role as a structural unit in lipid droplets, but also for the absorption and transport of carotenoids. However, further research is warranted to establish direct correlation between intracellular levels glycerophosphocholine and the absorption of target nutrient nutrients.

Intracellular levels of my-inositol were also found to be significantly altered by treatment groups and there was a significant interaction ($p = 0.0169$) between treatment group and time of exposure. Results showed that myo-inositol levels tended to decrease over time in moringa treatment where the opposite trend was observed for baobab treatment. Baobab treatment at day "10" resulted a significantly higher levels of myo-inositol as compared to moringa treatment for the same time point. Despite the declining levels of myo-inositol observed with moringa treatment, the changes were not significantly different from control. Myo-inositol is involved in a broad array of cellular functions particularly pertaining to its role in signal transduction as a precursor to inositol phosphates and phosphoinositides (253). Additionally, myo-inositol serves as an osmolyte within the cell where it has been shown to prevent effects associated with high ionic concentrations. Studies have demonstrated that hypotonic stimulus triggers the release of organic osmolytes such as taurine and myo-inositol to protect cells from potential swelling-induced cell lysis (254). Furthermore, paracellular permeability of Caco-2 cells has been shown to be modified by hyperosmosis induced by the addition of osmotic regulators highlighting the importance of maintaining cellular osmolarity for nutrient transport across the enterocytes (255). The increase in myo-inositol levels observed particularly at day 10 of baobab treatment as compared to moringa treatment indicates that baobab may facilitate the intracellular production and release of myo-inositol to protect cells from the increase in volume that occurs during the differentiation process as it has been previously reported (256).

Table 4-2 Major intracellular metabolites that showed significant changes in Caco-2 cells collected at 0, 5 and 10 of days of differentiation with chronic treatment of control, moringa or baobab 1,2,3

		Metabolites				
	Treatment	**glycerophosphocholine**	**taurine**	**myoInositol**	**AMP**	**PNP**
Day "0"	Control	4.91 ± 0.92c	0.99 ± 0.02c	0.43 ± 0.02a	0.07 ± 0.02	0.06 ± 0.02bc
	Moringa	3.97 ± 0.46d	0.9 ± 0.06d	0.48 ± 0.05ab	0.05 ± 0.02	0.06 ± 0.01bc
	Baobab	5.58 ± 0.2c	1.05 ± 0.39c	0.35 ± 0.15c	0.05 ± 0.01	0.05 ± 0.01c
Day "5"	Control	6.97 ± 0.54ab*	1.57 ± 0.11*	0.45 ± 0.05abc	0.04 ± 0	0.07 ± 0b
	Moringa	4.5 ± 0.32b*	1.36 ± 0.08*	0.39 ± 0.02bc	0.05 ± 0.01	0.06 ± 0bc
	Baobab	6.41 ± 0.22a*	1.44 ± 0.03*	0.43 ± 0.03abc	0.05 ± 0.01	0.06 ± 0bc
Day "10"	Control	6.89 ± 0.63ab*	1.69 ± 0.05*	0.43 ± 0.08abc	0.04 ± 0	0.07 ± 0.02b
	Moringa	4.68 ± 0.41b*	1.42 ± 0.27*	0.37 ± 0.06c	0.05 ± 0	0.09 ± 0.01a
	Baobab	6.41 ± 1.2a*	1.82 ± 0.25*	0.51 ± 0.04a	0.04 ± 0	0.09 ± 0.01a

[1]Changes in metabolite concentrations during Caco-2 cells differentiation chronically treated with aqueous fraction of digested baobab, moringa or blank saline (control) were monitored. [2]Data are presented as mean ± standard error of the mean (SEM). Significant differences were analyzed using Student t-test. [3]Different letter indicate significant differences ($p < 0.05$) between treatment groups within metabolites and (*) indicates significantly higher levels of metabolite between the different time points within individual metabolites.

Interaction effects were also observed with treatment group and stage of differentiation for intracellular levels of purine nucleoside phosphorylase (PNP). Both baobab and moringa treatment resulted in increased levels of PNP over the course of the differentiation period. At day 10 of differentiation, intracellular PNP levels with baobab and moringa treatment were found to be significantly higher than control at the same time point. Interestingly, the control treatment did not result in significant alterations of PNP levels during the differentiation period. Contrary to intracellular levels of glycerophosphocholine, myo-inositol and PNP, levels of taurine and (adenosine monophosphate) AMP were not significantly affected by repeated exposure to baobab or moringa as compared to the control treatment. Taurine levels seemed to significantly increase after day 5 of differentiation for all treatment groups indicating enzymes involved in its biosynthesis are only expressed in mature, fully differentiated Caco-2 cells as indicated by Lee et al (247). Overall, the current study provides some evidence that specific metabolites can be altered by repeated exposure to mineral-rich baobab and moringa ingredients over the course of cellular

differentiation and suggest that subsequent intestinal cell function pertaining to nutrient absorption may be impacted.

4.3.2 Carotenoid accumulation by Caco-2 cell monolayers is impacted by repeated exposure to digesta during differentiation

To further explore the consequences of the changes in cellular differentiation, a parallel study to investigate the effects of repeated exposure to mineral-rich baobab and moringa on the acute uptake and intracellular accumulation of carotenoids was investigated. Micellar aqueous fraction generated from digested carrot samples (with 5% oil) were diluted with DMEM and added to differentiated Caco-2 monolayers that were either exposed acutely or through differentiation to aqueous fraction of digested baobab, moringa or saline (control) and incubated for 4 h at 37 °C. Carotenoid content of treatment media as well as intracellular content post-incubation period is presented in Table 2. **Figure 4.3** (α-carotene) and **4.4** (β-carotene) show the uptake efficiency of Caco-2 cells as determined by the ratio of Caco-2 carotenoid intracellular content to that of the aqueous fraction content within treatment media The main carotenoids identified in both treatment media and in treated Caco-2 cells were all-trans-β-carotene and α-carotene as shown in **Chapter 3** and previous reports from digested carrot samples (257–259). The amount of α-carotene and β-carotene in treatment media ranged from 166 – 169 pmol/well and 203-210 pmol/well respectively. After 4 h of incubation, intracellular content of α-carotene with acute exposure of control, baobab, or moringa was found to be 14.7 ± 0.9, 13.8 ± 1.7, or 14.9 ± 0.7 9 pmol/mg of protein respectively with no significant differences between treatment groups. While there was a significant reduction ($p < 0.05$) in intracellular content of α-carotene in monolayers having been exposed through differentiation compared to acute treatment for control, baobab or moringa.

Table 4-3 carotenoid profile of treatment media and Caco-2 cells after 4h incubation with treatment media 1,2

Treatment	Treatment media (pmol/well)		Cellular content (pmol/ mg of protein)			
			α-carotene		β-carotene	
	α-carotene	β-carotene	acute	chronic	acute	chronic
Control	166.3 ± 10.4	203.8 ± 13	14.7 ± 0.9a	7.7 ± 0.5b	19.4 ± 0.8a	12 ± 0.6b
Baobab	161.4 ± 8.3	196.7 ± 11	13.8 ± 1.7a	7.5 ± 0.4b	17.9 ± 1.7a	11.3 ± 0.6b
Moringa	169.2 ± 7.8	210 ± 9.7	14.9 ± 0.7a	7.2 ± 0.6b	19.5 ± 0.9a	10.8 ± 0.8b

[1]Caco-2 cells chronically or acutely treated with aqueous fraction of digested baobab, moringa or blank saline were incubated for 4 h with micellar fraction generated from digested carrots (with 5% oil). [2]Data are presented as mean ± standard error of the mean (SEM). Significant differences were analyzed using Student's t-test. Different letter indicate significant differences ($p < 0.05$) between acute vs. chronic treatments within individual carotenoid species. No significant differences ($p > 0.05$) were observed between treatment media within each carotenoid species.

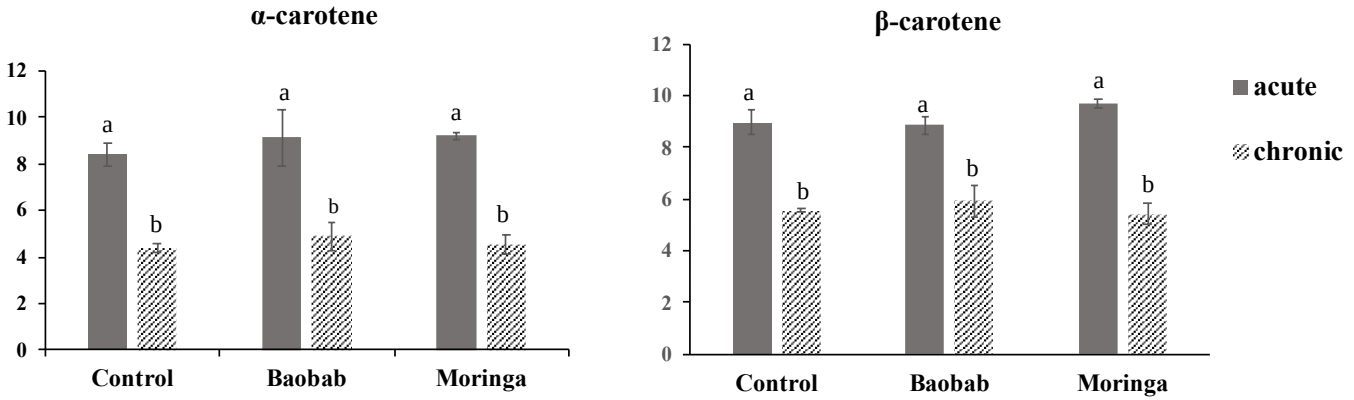

Figure 4.3 Uptake efficiency by Caco-2 monolayers of α-carotene from acute (solid bar) and chronic (striped bar) of control, baobab or moringa treatment. 1Each data point represents mean ± standard error of percent ratio of total cellular carotenoid content to that of the aqueous fraction (n=4 wells at each time point). Significant differences ($p < 0.05$) are designated as different letters.

The decrease in α-carotene levels observed with baobab or moringa treatment was not significantly different from control digesta exposure. Similar findings were observed for intracellular β-carotene content where there were no significant differences between acute exposure of baobab or moringa as compared to the control with levels ranging 17.9 – 19.5 pmol/mg of protein. β-carotene absorption was significantly reduced in Caco-2 cell monolayers differentiated with baobab, moringa or control however, there were no significant differences between these pretreated groups highlighting that the effect observed during repeated treatment is not due to the presence of the mineral-rich plant materials. This suggests that there are factors within the simulated gastrointestinal digesta itself separate from treatment samples that may modulate nutrient uptake. Most Caco-2 cell studies investigating nutrient absorption and transport use growth media as a control and do not account for intracellular changes that may occur due to the exposure of the digesta matrix. The present study, while suggesting that repeated exposure to plant extracts may not alter carotenoid uptake, does provides important insight into the effect of the background digesta matrix on nutrient uptake and offers insights for future studies to consider this factor when evaluating test samples using Caco-2 cell models.

4.3.3 Impact of chronic exposure to digesta on carotenoid transporters

In order to better understand the reductions in carotenoid accumulation from exposure to digesta, changes in the expression of genes involved in the transport of carotenoids as well as minerals (iron in particular) were explored. Beyond passive diffusion, carotenoid absorption by the enterocytes is believed to be mediated by transporter proteins including Scavenger Receptor Class B type 1 (SR-B1), Cluster Determinant 36 (CD-36) and Niemann-Pick C1-Like 1 (NPC1L1) (244,260,261). Fatty Acid Binding Protein 1 (FABP1) has also been proposed to participate in carotenoid absorption due its broad specificity (262). In the present study, mRNA levels of SR-

B1, CD36 as well as FABP1 were measured following acute exposure of differentiated Caco-2 monolayers to carrot aqueous digesta either with our without pre-exposure to mineral-rich baobab, moringa or 0.9% saline (control) during the extent of differentiation (10 days). mRNA levels of SR-B1 from non-pretreated Caco-2 cells acutely treated with baobab or moringa were not found to be significantly different ($p > 0.05$) from control group (**Figure 4.4**). Comparison of acutely and chronically treated cells also revealed that while SR-B1 and FABP1 mRNA levels tended to be lower in all treatment groups with chronic exposure, there were no significant differences between exposure length for all treatment groups. The trend in decline of these transporters is in alignment with reduced carotenoid absorption observed with chronic exposure of all treatment groups (**Figure 4.3**). mRNA levels of CD36 did not show significant differences between treatment groups for acute or chronic treatment. This finding is in contrast to the observed reduction of carotenoid uptake described earlier indicating that the transporters, particularly CD36, might not be as responsive to changes in carotenoid levels in the intracellular environment. While several studies have investigated the role of CD36 role in carotenoid absorption *in vivo* and *in vitro*, recent reports have demonstrated that CD36 may be less sensitive to changes in carotenoid levels within Caco-2 cells as it has been shown that inhibition of CD36 did not affect the transport of β-carotene (261). Additionally, mRNA levels of CD36 in Caco-2 cells have been shown to be 4% of the level found in human intestinal cells providing further insight for the lack of differences observed in the current study as well as previous studies (261,263).

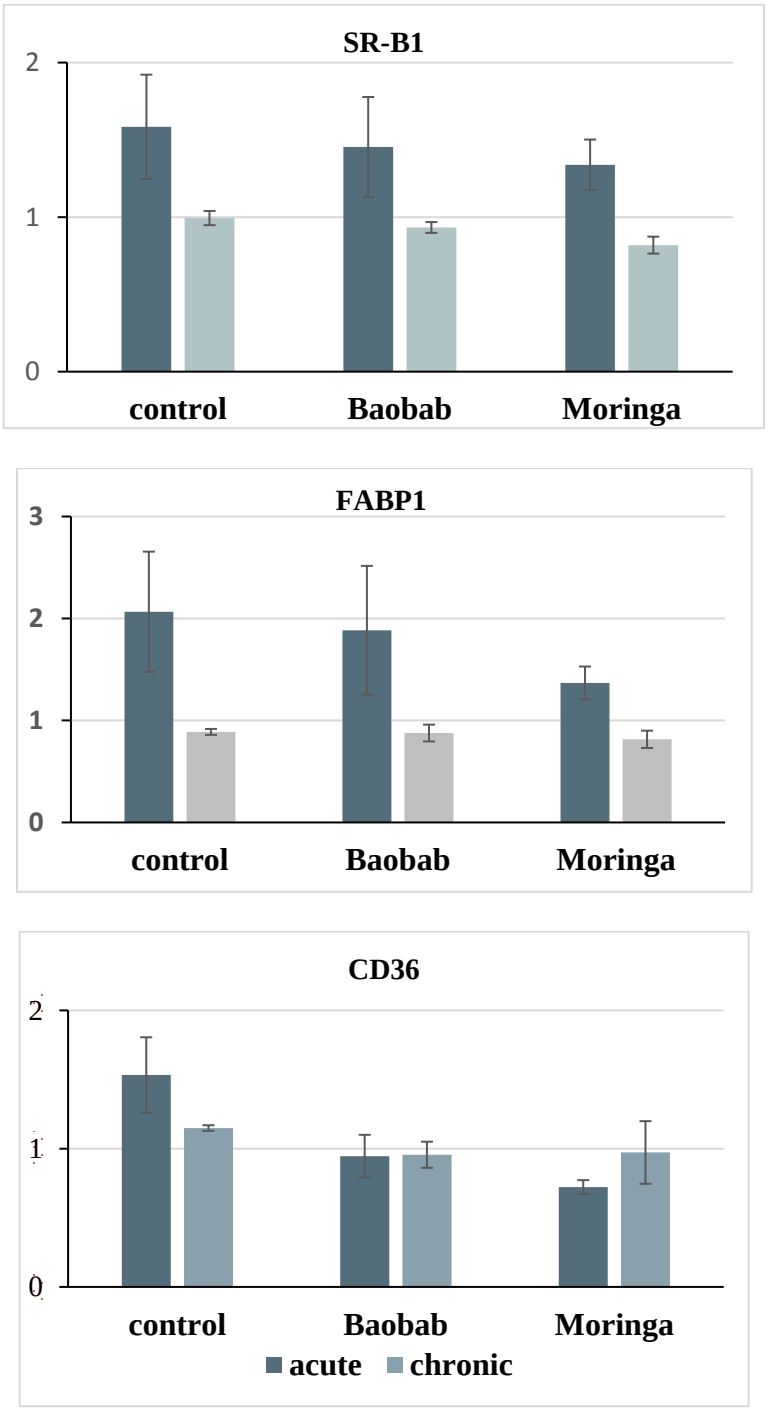

Figure 4.4 1Data are presented as mean ± standard error of the mean (SEM). Significant differences were analyzed using Student Tukey's HSD test. No significant differences ($p > 0.05$) were observed in mRNA expression of SR-B1, FBP1 and CD36 between treatment groups.

Despite the lack of significant alterations in mRNA levels with acute and chronic exposure of all treatment groups, significant reduction ($p < 0.05$) in SR-B1 protein levels were observed in chronic exposure as compared to acute treatment particularly in control (0.9% saline) samples (Figure 5). However, there were no significant differences in SR-B1 protein levels between acute and chronic exposure within baobab or moringa treatment. The reduction in SR-B1 protein levels in control sample is in agreement with our findings in the carotenoid uptake experiment showing a reduction in carotenoid absorption with chronic exposure of all treatment groups including control samples.

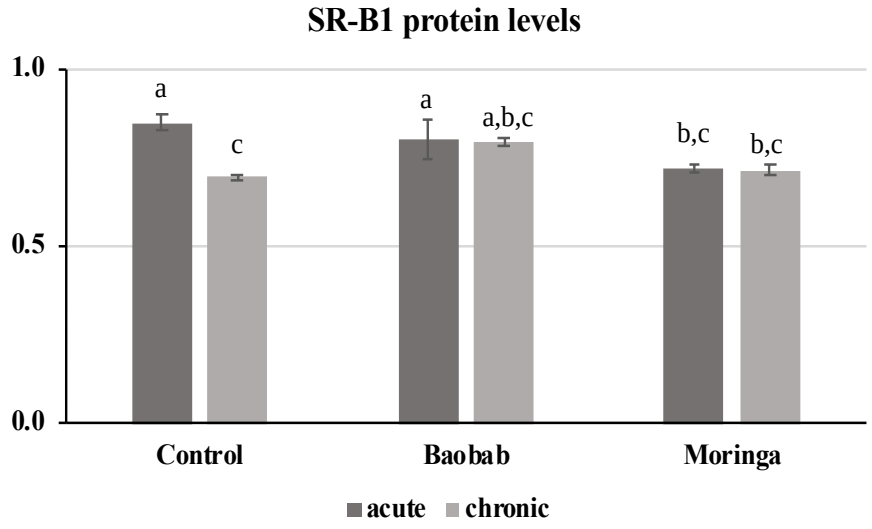

Figure 4.5 1Data are presented as mean ± standard error of the mean (SEM). Significant differences were analyzed using Student's t-test. Different letter indicate significant differences ($p < 0.05$) in SR-B1 protein levels among the different treatment groups.

A potential cause for the decrease in SR-B1 levels and thereby decrease in carotenoid uptake with chronic exposure maybe due to the build-up of digestion by-products particularly bile acids that may modulate the expression of transporter genes to protect cells from potential toxicity. The expression of SR-B1 has been shown to be regulated by bile acids both in cell and animal models (264,265). Malerod et al. (2005) demonstrated that bile acids limit their own biosynthesis within hepatocytes to prevent potential overload by a mechanism that initiates a nuclear cascade

leading to the down-regulation of SR-B1 expression thereby inhibit cholesterol supply into the hepatocyte (265). The lack of significant reduction in SR-B1 protein levels with exposure of baobab or moringa over differentiation compared to those observed in control treatments may be due to the presence of high divalent minerals present in these plants that may bind bile acids thereby inhibiting their effects on SR-B1 expression. Overall, the current findings suggest that, at the levels introduced in this study which match relevant levels that could be implemented in consumer products, mineral-rich baobab or moringa may only have a modest impact on intestinal absorption of carotenoids and the expression of genes involved in carotenoid transport within the small intestine. However, *in vivo* studies confirming such findings are warranted.

4.4 Conclusion

While the use of these nutrient dense indigenous plant materials presents a potentially economically viable path to providing adequate levels of short fall micronutrients including iron and vitamin A, the impact of incorporating such materials into daily food habits on nutrient absorption and metabolism is unknown. In the present study we investigated the impact of chronic exposure to baobab and moringa at levels that would constituent 15% of the dry matter in a cereal porridge on Caco-2 human intestinal cell differentiation and provitamin A uptake. This level was chosen as it represents a level that could deliver ~15% RDA for iron. Intracellular metabolites identified by NMR as markers of Caco-2 cell differentiation with chronic exposure to digested baobab and moringa samples indicated an apparent clustering of baobab and control treatment differentially from moringa treatment particularly at day 5 of differentiation. Evaluation of individual metabolites suggested modest differences in differentiation by treatment groups. Overall, there was an increase in specific osmotic pressure regulators (glycerophosphocholine, taurine and myo-inositol) for all treatment groups except for myo-inositol levels with moringa

treatment. Alteration in levels of these metabolites have been associated with specific cellular function including protection against hyperosmotic stress and regulation of paracellular permeability of Caco-2 cells. Therefore, the increase in these metabolites observed with chronic exposure to all treatment groups could be a potential mechanism to protect cells from excess accumulation of the digesta matrix which may in turn regulate nutrient uptake as observed with overall reduction of carotenoid absorption with chronic exposure to all treatments.

Carotenoid uptake as well as expression of genes involved in carotenoid transport were evaluated with and without chronic exposure of baobab and moringa during differentiation. Overall, there were no significant differences in carotenoid uptake with acute or chronic treatment between treatment groups although chronic treatment of all treatment group including the control resulted in significant reduction. As mentioned earlier, the overall decrease in carotenoid absorption with repeated exposure of all treatment groups may be due to presence of increased levels of digesta material accumulating within the cells which may trigger the intracellular release of metabolites (osmolytes) that regulate cellular function related to nutrient absorption. mRNA levels of carotenoid transporters, mainly CD36, FBP1 and SR-B1 were not significantly altered by acute or chronic treatment of plant materials although FBP1 and SR-B1 mRNA levels tended to decrease with chronic exposure. Despite the lack in significant changes of mRNA levels, there was a significant decrease in SR-B1 protein levels particularly in control treatment. Reduction in SR-B1 levels may be a potential mechanism to protect cells bile acid accumulation from the digesta matrix as it has been shown that bile acids regulate their own biosynthesis by a mechanism that involves the down-regulation SR-B1 expression. This finding offers an additional insight into the cause of carotenoid uptake reduction with chronic exposure separate from the findings from the metabolomic analysis. However, further *in vitro* and *in vivo* studies are warranted to elucidate the

underlying mechanism by which gastrointestinal fluids (separate from test samples) may regulate carotenoid absorption. Overall, the results indicated that, at the levels introduced in this study which were designed to match levels to that could be incorporated into consumer products, mineral-rich baobab or moringa did not cause significant alterations in carotenoid absorption by Caco-2 cells. This suggests that such combined formulations leveraging baobab and moringa may not conflict in their objective to deliver both iron and provitamin A carotenoids.

CHAPTER 5. CONCLUSION AND FUTURE DIRECTIONS

The integration of underutilized fruits and vegetables into daily diets has been shown to be one of the most practical and sustainable ways to overcome vitamin A deficiency in Sub-Saharan Africa. Leveraging these plant materials at harvest or second quality products not suitable for fresh markets, can provide households with raw materials rich in micronutrients for application in daily preparation or as commercial ingredient for new processed foods. However, in order to best leverage their potential quality to meet vitamin A requirements, additional insight into their nutritional composition and knowledge on the bioaccessibility and bioavailability of provitamin A carotenoids from such foods incorporated into staple food preparation methods is required. Therefore, the works of this dissertation were aimed at addressing knowledge gaps of thorough nutritional profile of native African plant materials and their potential impact on provitamin A carotenoid bioaccessibility from blended cereal-based product formulations using *in vitro* models. The success of food based strategies is dependent upon the habitual intake of these underutilized plant materials and their incorporation into existing food consumption patterns. As a result, we also investigated the impact of long-term exposure to these nutrient dense plant materials on provitamin A carotenoid absorption and monitored any biochemical alterations in intestinal cell differentiation using Caco-2 human intestinal cell model.

Our findings discussed in **Chapter 2** provided comprehensive characterization of phytochemicals as well as polysaccharides from local plant materials, particularly Adansonia digitata (baobab) fruit, Moringa Oleifera (moringa) leaves and Hibiscus Sabdariffa (hibiscus) flowers. Results from this study will provide important information on key bioactive compounds that could be targeted for their use as functional and nutritional food ingredients in product formulations. Specific to their use as sources of provitamin A carotenoids, our findings showed

that while hibiscus and baobab contained minimal levels, moringa leaf powder had appreciable levels of β-carotene and α-carotene. However, the levels found in our study were substantially lower than those previously reported. Although the cause of such significant loss is unclear, we proposed that the decline of carotenoid levels may be due to post-harvest handling and processing conditions used in preparation of moringa powder during commercial production. Therefore, studies focused on optimization of processing conditions should be conducted to improve retention of provitamin A carotenoids in these plant materials. Furthermore, as we hypothesized, we noted these plants contained specific bioactive compounds that may directly or indirectly modulate the bioavailability of provitamin A carotenoids as well as other micronutrients such as iron and zinc. Further studies are warranted to investigate the impact of such compounds isolated from these plant materials on the bioavailability of target nutrients. Information gained from the polysaccharide composition of baobab, moringa and hibiscus could be leveraged to design potential functional ingredients as emulsifiers for the delivery of provitamin A carotenoids. The presence of high proportions of xyloglucans in baobab may explain its traditional use as a thickening agent. Hibiscus was found to contain similar proportions of xyloglucans and pectic polysaccharides (AG-I, AG-II and RG-I) whereas moringa was found to be primarily composed of pectic polysaccharides. We proposed that the primary pectic polysaccharide in moringa (AG-II) could be found in the form of arabinogalactan proteins complexes (AGP) which are known to be strong emulsifying agents. However, further structural elucidation using NMR spectrometry is needed to confirm the presence of such polysaccharides. Additionally, the effect of the specific polysaccharides identified from moringa, hibiscus and baobab on provitamin A carotenoid bioavailability needs to be established. Some soluble (pectin, guar gum, and alginate) and insoluble fibers (cellulose) have been reported to hinder carotenoid absorption by precipitating and

increasing the excretion of bile salts. However, these studies were mostly carried out on pure polysaccharide isolates and not from those complexed in their food matrices. The limiting effects of polysaccharides observed in pure isolates could be counteracted by the presence of other bioactive compounds from whole food matrices. Therefore, *in vivo* studies evaluating the impact of these plant materials as a whole and their isolated polysaccharides/bioactive compounds would provide valuable insight into their role as nutritional and functional ingredients.

In **Chapter 3**, we investigated the incorporation of baobab as a natural iron fortificant (both as a source and enhancer of iron bioavailability), on the bioaccessibility of provitamin A carotenoids from dried carrot and mango blend co-formulated in frequently consumed millet-based porridge meals. The incremental incorporation of baobab into porridge formulations seemed to limit the micellarization of carotenoids but not their intestinal uptake by Caco-2 cell monolayers. The exact mechanism by which baobab may interfere with the incorporation of carotenoids into mixed micelles is unknown. However, we propose that high mineral levels, particularly divalent minerals such as calcium and magnesium in baobab may play a role as their presence has been shown to alter physiochemical parameters of micelles by binding with fatty acids and bile salts. Additionally, the specific phenolic (flavan-3-ols) and polysaccharide (xyloglucans) profile of baobab identified in **Chapter 2** may play a role in the observed reduction of carotenoid micellarization. Further studies evaluating the impact of isolated baobab components (minerals, phenolics and polysaccharides) on the physical properties of fluids within the digestive system need in order to elucidate the underlying mechanism by which baobab may modulate carotenoid micellarization. Nonetheless, our observations from intestinal uptake study were encouraging as carotenoid absorption was not affected by the presence of baobab. The mechanism by which baobab may counteract its own limiting effect observed during micellarization of carotenoids was

not established. It is possible that the presence of organic acids in baobab my provide stability to the carotenoids during their accumulation within the Caco-2 cells as citric acid has been shown to promote thermal stability of β-carotene. While there is also the possibility that baobab may upregulate the expression of genes involved in carotenoid absorption, our findings in **Chapter 4** showed that mRNA and protein levels of carotenoid transporters (SR-B1 and CD36) from Caco-2 cells were not significantly altered by acute and chronic treatment of baobab at the levels introduced in that study.

Lastly, as presented in **Chapter 4**, we investigated the impact of long-term exposure of nutrient-dense plant materials (baobab and moringa) on the absorption of provitamin A carotenoids as well as cellular differentiation biomarkers of Caco-2 cell monolayers. Generally, our findings showed that the introduction of baobab and moringa at levels that could be incorporated into consumer products (used as mineral sources or enhancers) did not cause any significant alteration in carotenoid absorption and the expression of carotenoid transporter genes. When comparing acute versus chronic exposure to baobab, moringa or control treatment (saline solution), there was an overall decline in carotenoid absorption with chronic exposure to all treatments including the control. This finding was interesting in that it highlighted that the reduction of carotenoid absorption during chronic exposure was not due to the presence of baobab or moringa but more so due to the continued accumulation of digestion by-products. This suggests that there are factors within the digesta matrix itself that may serve as feedback mechanism to regulate excessive exposure to nutrients that directly or indirectly affect carotenoid uptake by the small intestine. For instance, bile salts are known to limit their own biosynthesis to prevent cellular toxicity by downregulating the expression of cholesterol and carotenoid transporter, SR-B1. Therefore, the repeated exposure to digesta material containing bile salts may inhibit carotenoid uptake by a

similar mechanism. Additionally, as demonstrated in the metabolomic analysis, the increase in osmotic regulators (glycerophosphocholine, taurine and myo-inositol) observed during cellular differentiation with chronic treatment indicates that the reduction in carotenoid uptake may be due to a cellular response mechanism to an overall hyperosmotic stress that is separate response from to cytotoxicity. Most studies investigating nutrient absorption and transport using Caco-2 cells use growth media as a control and do not account for intracellular changes that may occur due to exposure to gastro-intestinal fluids separate from the test treatments. Our study provides interesting insight into the effect of digesta matrix alone on nutrient uptake and cautions future studies to consider this effect when evaluating test samples using Caco-2 cell models.

The collective findings from this dissertation work indicate that the incorporation native African plant materials rich in minerals and other bioactive compounds did not significantly affect the ultimate delivery (i.e. absorption) of provitamin A carotenoids at the levels introduced in the present study as assessed by *in vitro* models. While these findings show promise for the use of combined product formulations targeted to address both vitamin A and minerals, *in vivo* studies are warranted to support our findings and determine the efficacy of these formulations to meet vitamin A requirements.

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APPENDIX A SUPPLEMENTARY FIGURES FOR CHAPTER 2

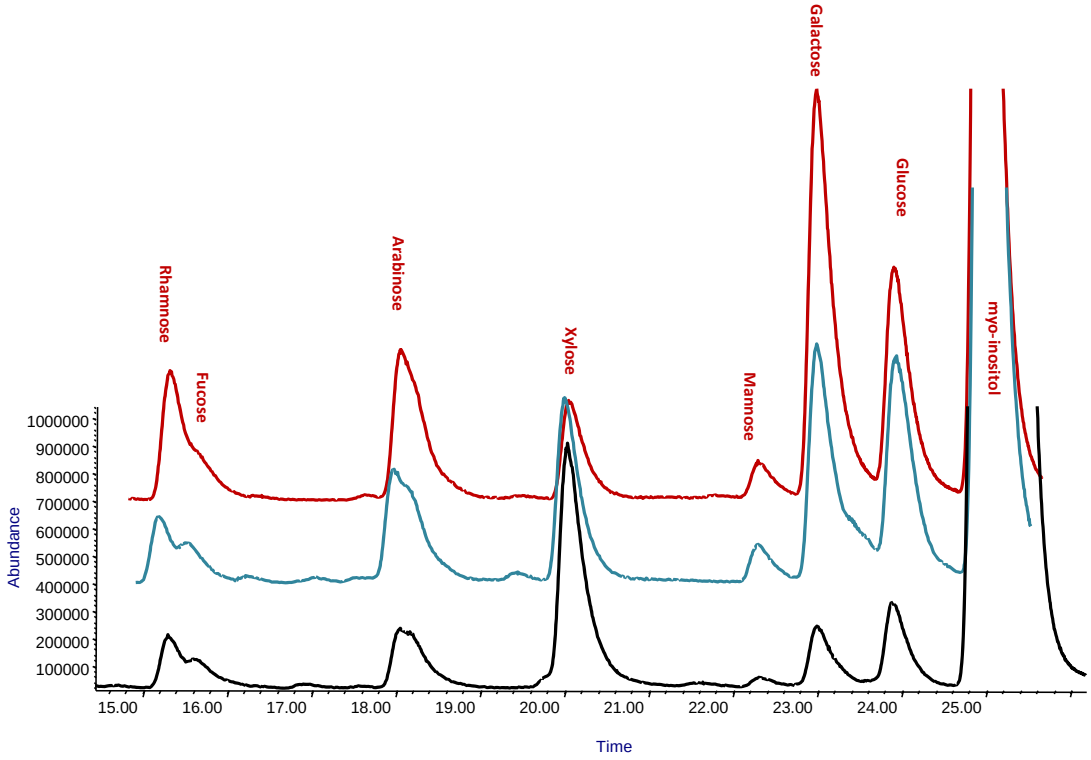

Figure A. 1 Chromatogram of monosaccharides identified from commercially available moringa leaf (red), hibiscus flower (blue) and baobab fruit powder quantified as alditol acetate derivatives

APPENDIX B SUPPLEMENTARY FIGURES FOR CHAPTER 2

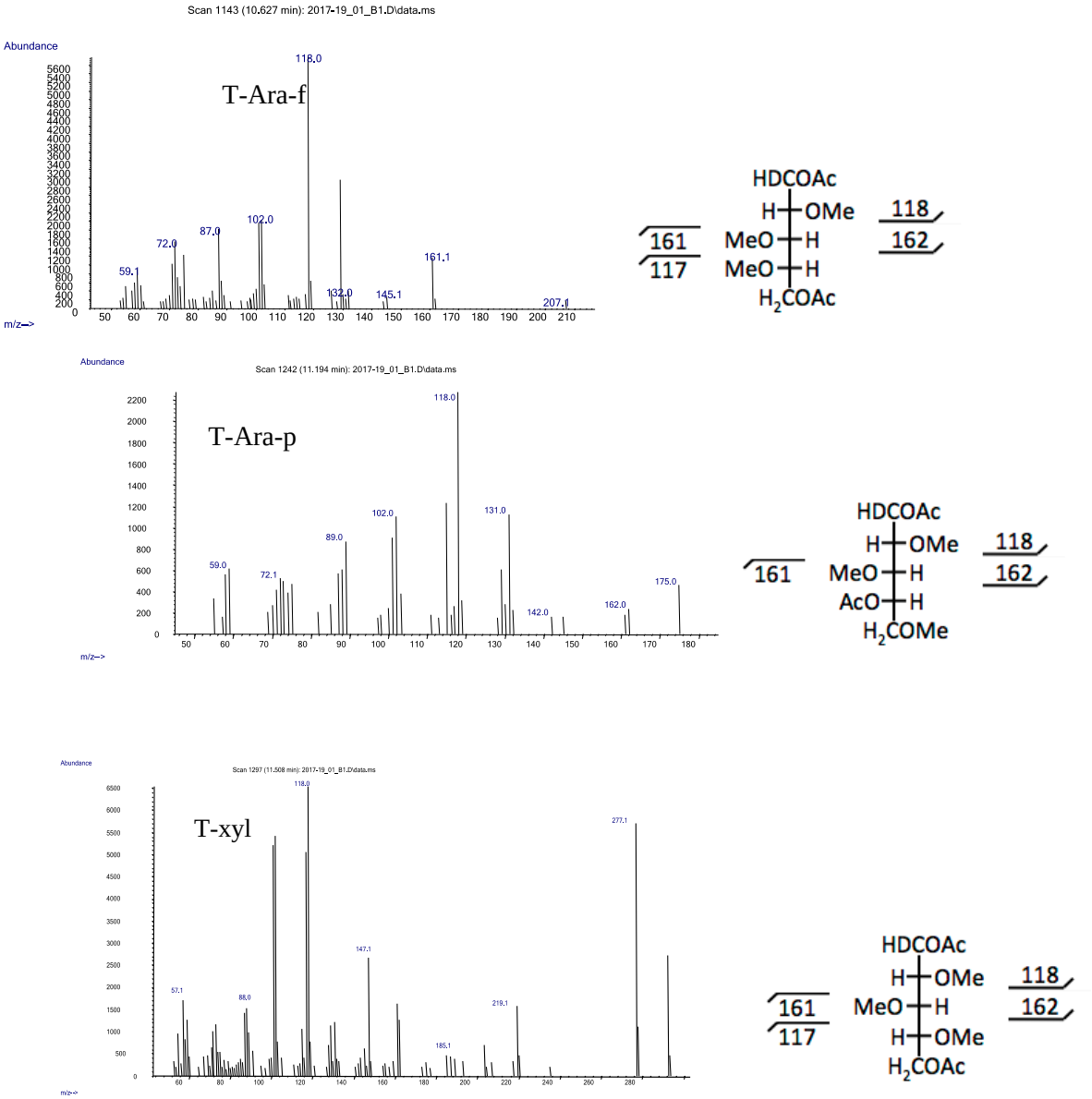

Figure B. 1 Mass spectrum and fragmentation pattern for the identification of glycosyl-linkage of monosaccharide residues by partially methylated alditol acetate derivatization

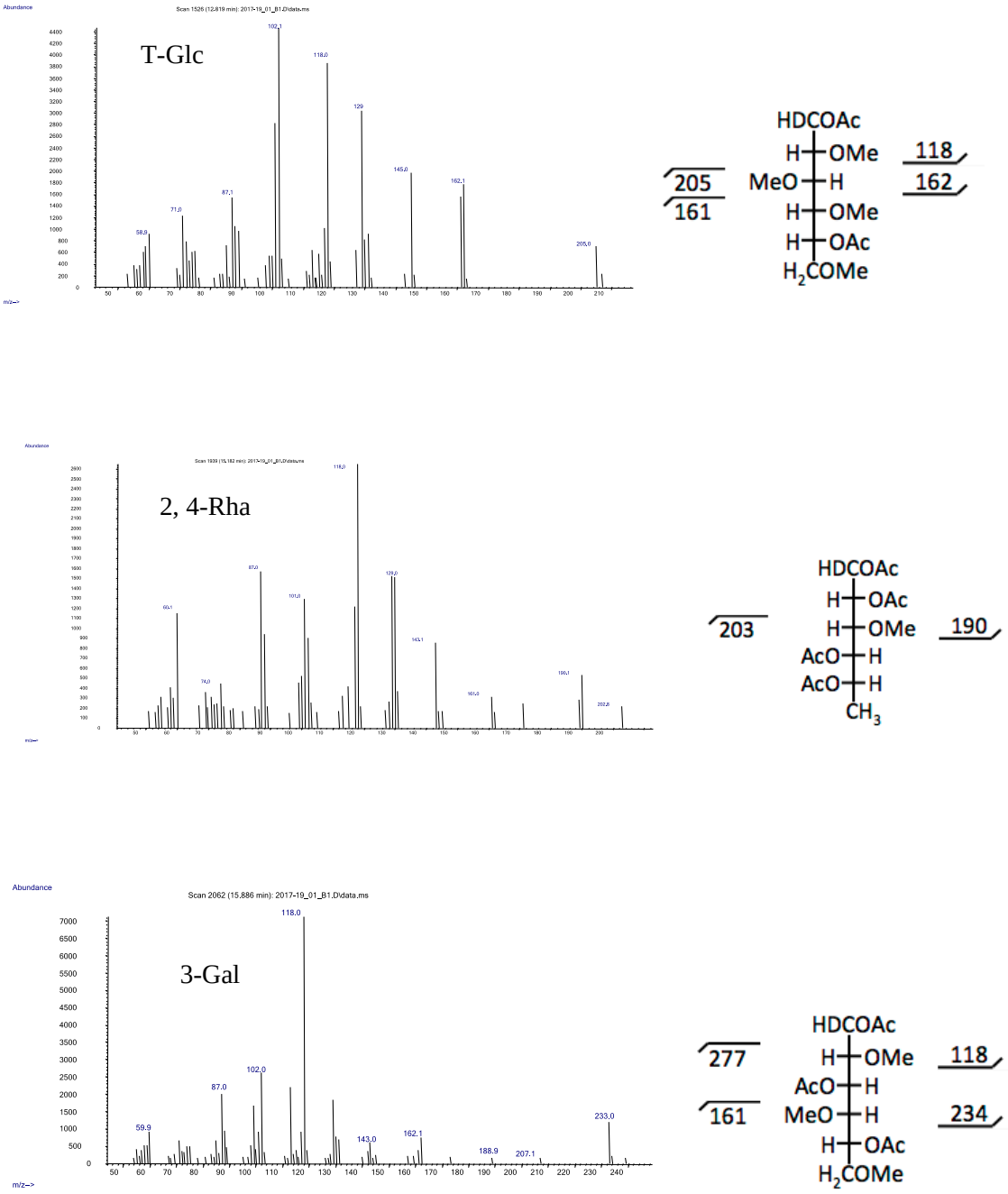

Figure B. 2 Mass spectrum and fragmentation pattern for the identification of glycosyl-linkage of monosaccharide residues by partially methylated alditol acetate derivatization

APPENDIX C SUPPLEMENTARY TABLES FOR CHAPTER 3

Table C. 1 Relative bioaccessibility of provitamin A carotenoids from porridge meals (µg/per serving)1,2

Sample	β-cryp	15-cis-βC	13-cis-βC	All-trans-αC	All-trans-βC	9-cis-βC	TBC	TPVA
Control	36.06a	23.13a	35.41a	24.05a	23.25a	25.89a	27.26a	23.58a
Baobab 5%	25.82ab	22.55ab	29.91ab	20.60ab	20.31ab	20.93ab	22.75ab	20.46ab
Baobab 15%	25.90ab	20.85ab	27.51ab	18.57ab	18.40ab	22.03ab	22.92ab	18.58ab
Baobab 25%	21.17b	15.97b	21.51b	13.21b	13.36b	12.59b	14.52b	13.38b

Percent micellarization efficiency (relative bioaccessibility) of individual carotenoid species from control, baobab at 5%, baobab at 15%, baobab at 25% digested porridge meals. [2]Abbreviations used: β-cryptoxanthin (β-CRP), all-*trans*-α-carotene (all-trans-αC), all-*trans*-β-carotene (all-trans-βC), *cis*-β-carotene isomers (cis-βC). [3]TPVA represents total provitamin A carotenoids calculated as β-carotene equivalent calculated as follows: all-trans-βC + 1/2(β-cryp + all-trans-αC + cis-βC). All data presented are expressed as mean ± standard error of the mean of n=4 analyses. Different letters indicated above the bars represent significant differences in micellarization efficiency (%) between porridge formulations within individual carotenoid species ($p < 0.05$).

Table C. 2 α- and β-carotene accumulation by Caco-2 cells (pmol/mg protein) 0, 2, 4 and 6hr of incubation with diluted micellar aqueous fraction from porridge meals containing 0, 5, 15 or 25% baobab[1]

	All-trans-αC				all-trans-βC			
Time (hr)	control	Baobab 5%	Baobab 15%	Baobab 25%	control	Baobab 5%	Baobab 15%	Baobab 25%
0hr	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± a0	1.2 ± 0.3a	1.1 ± 0.2a	0.7 ± 0.2a	0.5 ± 0.2a
2hr	4.5 ± 1.4b	3.0 ± 0.9b	2.1 ± 0.7b	1.6 ± 0.7b	7.3 ± 1.8b	5.8 ± 1.4b	4.5 ± 1.1b	3.9 ± 1.1b
4hr	8.0 ± 0.8c	6.3 ± 0.8cd	4.5 ± 0.7ef	3.1 ± 0.8d	14.0 ± 1.6c	11.1 ± 1.5cd	8.4 ± 1.4cd	6.4 ± 1.5d
6hr	12.3 ± 2.5e	10.9 ± 1.6ef	7.3 ± 0.8ef	4.4 ± 0.6f	21.1 ± 4.4e	19.1 ± 3.0ef	13.2 ± 1.5ef	8.7 ± 1.2f

α-carotene and β-carotene accumulation by Caco-2 cell monolayers incubated with control, baobab 5%, baobab 15%, baobab 25% treatment samples for 0, 2, 4 and 6hr. [1]Each data point represents mean ± standard error of percent ratio of total cellular carotenoid content to that of the aqueous fraction (n=4 wells at each time point). Different letters represent significant differences between treatment within the same time point ($p < 0.05$).