Measuring provitamin A carotenoids and their retinyl ester bioconversion products using high-performance liquid chromatography coupled with high-resolution mass spectrometry: Application to bioavailability studies in humans

by

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The student author, whose presentation of the scholarship herein was approved by the program of study committee, is solely responsible for the content of this dissertation. The Graduate College will ensure this dissertation is globally accessible and will not permit alterations after a degree is conferred.

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ABSTRACT

Vitamin A deficiency remains prevalent in South Asia and sub-Saharan Africa. The well-established vitamin A supplementation program in children virtually reduces xerophthalmia and mortality in children but offers only a transient impact on raising serum retinol. Regular consumption of vitamin A-fortified food is considered a sustainable approach to maintain a healthy serum response of retinol of vulnerable populations. Bananas dominate the diet of many East-African countries where vitamin A deficiency is prevalent, thus making it a great vehicle for biofortification, a breeding technique to improve vitamin A value in the crop and increase dietary intake of vitamin A. Currently, provitamin A carotenoid (α- and β-carotene) biofortified bananas are being developed through genetic modification but its bioefficacy in humans has not been studied. The dominant provitamin A found in the biofortified bananas is α-carotene, followed by β-carotene. The nonsymmetrical α-carotene provides a molecule of active vitamin A (retinol) and its inactive analog (α-retinol). Exclusion of the inactive α-retinol with limited vitamin A activity is important for accurate determination of vitamin A activity of α-carotene in α-carotene containing foods such as provitamin A biofortified bananas. The overall objective of this study was to accurately determine the bioconversion factor of the α-carotene-containing provitamin A biofortified bananas in humans after the consumption of provitamin A biofortified bananas. An ultra-high selective and sensitive high-performance liquid chromatography–quadrupole-time-of-flight–high-resolution mass spectrometric with electrospray ionization in positive mode (HPLC-ESI (+)-QTOF-HRMS) quantitative method was developed and applied to the measurement of the postprandial appearance of provitamin A carotenoids and their retinyl ester bioconversion products as well as α-retinyl palmitate product of α-carotene after the consumption of provitamin A biofortified bananas. A 3µm C30 carotenoid column was used to
separate α- and β-carotene, and their respective retinyl ester bioconversion derivative of α-retinol and retinol (i.e., α-retinyl ester and retinyl ester) in postprandial plasma triacylglycerol-rich lipoproteins. Labeled internal standards (d₈-α-retinyl palmitate, ¹³C₁₀-β-carotene, ¹³C₁₀-retinyl palmitate) were used to account for analysis variability. Twelve healthy women each consumed three 200-g cooked banana fruit as follows: 1) biofortified banana fruit containing 1415.3 µg (2.64 µmol) of total β-carotene equivalents, 2) wild-type banana fruit with a β-carotene reference dose containing 536.8 µg (1.00 µmol) added β-carotene, and 3) wild-type banana fruit with a vitamin A reference dose containing 262.5 µg retinol activity equivalents (0.92 µmol) added retinyl palmitate. Mean (±SD) areas under the curve for retinyl palmitate in the TRL fractions (nmol·h) were 67.2 ± 50.5, 167.3 ± 114.5, and 167.8 ± 111.5 after consumption of the provitamin A-biofortified banana fruit, the wild-type banana fruit with the β-carotene reference dose, and the wild-type banana fruit with the vitamin A reference dose, respectively. The vitamin A equivalence of provitamin A of biofortified bananas and of wild-type banana fruit with the β-carotene reference dose were 18.52 ± 10.9 µg (mean ± SD) and 2.29 ± 0.93 µg, respectively. The vitamin A equivalency of provitamin A carotenoid biofortified banana fruit was similar to that reported for carotenoid-rich vegetables such as carrots or spinach, but less than that reported for starchy matrices (e.g., cassava, maize and rice).
CHAPTER 1. GENERAL INTRODUCTION

Vitamin A is an essential fat-soluble micronutrient that is critical in the development of physiological functions of all vertebrates in life (1). Two available forms of vitamin A found in the human diet include preformed vitamin A (e.g., retinyl esters) from animal products, and provitamin A carotenoid (e.g., α- and β-carotene) richly found in colored fruits and vegetables. Vitamin A deficiency has been declared as a public health problem (2); it increases the risk of infectious disease specifically in preschool-age children, resulting in an increased risk of morbidity and mortality. Vitamin A deficiency continues to be the leading cause of preventable blindness in children. Pregnant women are at risk of night blindness, with an increased risk of maternal mortality caused by vitamin A deficiency.

A 2013 population-based survey estimated that the burden of vitamin A deficiency remains persistently high in South Asia and Sub-Saharan Africa, indicating an unchanging trend over the past two decades (3). This persistent high prevalence may be attributed to the lack of dietary diversification and unsuccessful vitamin A supplementation. More than 30% of children at risk are not receiving the health benefit of vitamin A supplementation (4). In addition, vitamin A supplementation offers only a transient improvement of vitamin A status of the vulnerable (5). Therefore, an alternative and complementary approach to the existing intervention includes increasing the consumption of vitamin A-fortified food products. This approach could help to eliminate vitamin A deficiency by providing adequate daily vitamin A intake.

Biofortification is a feasible plant breeding process to improve the nutrition quality of a food crop through traditional cross-breeding or modern technology by means of providing long-term sustainable dietary essential nutrients to improve quality of life (6). Because diets of the poor in most low-income countries rely heavily on starchy staples low in vitamin A and other
micronutrients, improving the nutritional values of starchy staple crops by biofortification is an effective way to increase daily adequacy of nutrient intakes (7). Nutrition evidence on the bioavailability and efficacy trials have suggested that consuming biofortified crops (e.g., maize, yellow-cassava and orange-flesh sweet potato) enriched with provitamin A carotenoid (e.g., β-carotene) is a sustainable strategy to improve vitamin A status by increasing serum β-carotene (8–10), thus complementing the existing interventions.

Bananas, including non-Cavendish and plantains, provide important household food security in many low-income African countries such as Uganda, Rwanda and Cameroon, where per capita annual consumption exceeds 200 kg of bananas (11). Considering its popularity in these countries, bananas are a sustainable and cost-effective vehicle for biofortification to deliver vitamin A and other micronutrients to at-risk populations. Because this fruit is triploid and sterile in nature, genetic modification is the only possible way to improve nutritional values. Genetic modification allows the accurate and precise re-engineering of the carotenoid biosynthesis pathway using the desirable gene from Fei’ banana cultivar and promoter to produce a provitamin A-rich banana cultivar (12). As part of the crop development process, the evaluation of bioavailability of the provitamin A enriched banana fruit is necessary to understand if the target provitamin A level in the newly developed crop is sufficient to make an impact to reduce vitamin A deficiency of the target populations (6).

Bioavailability of provitamin A-rich biofortified banana fruit in humans can be evaluated by defining its vitamin A equivalence, where the vitamin A activity of α- and β-carotene are determined after the ingestion of biofortified banana fruit. While the symmetrical β-carotene provides two molecules of retinol (vitamin A) at central cleavage, the asymmetrical α-carotene is cleaved to produce retinol (vitamin A) and its biological inactive α-retinol (non-vitamin A). The
vitamin A activity of $\alpha$-carotene is approximately half of the $\beta$-carotene value (13). Recently, Cooperstone et al. (14) described the large interindivdual variability of $\alpha$-carotene metabolism after the participants consumed a carrot meal. Their findings highlighted the importance of accurate quantitation of $\alpha$-carotene contribution to vitamin A activity in $\alpha$-carotene-containing foods. To avoid overestimation of vitamin A activity from $\alpha$-carotene-containing biofortified bananas, we seek to develop an ultra-high selective and sensitive high-performance liquid chromatography–high-resolution mass spectrometric (HPLC-HRMS) instrumentation as a quantitative platform to measure the postprandial appearance of $\alpha$- and $\beta$-carotene and the retinyl esters bioconversion products as well as $\alpha$-retinyl esters products of $\alpha$-carotene.

High-resolution mass spectrometry (HRMS) technology, which includes quadrupole time-of-flight (Q-TOF) and Orbitrap, continues to evolve as a state-of-the-art instrumentation that provides enhanced performance in molecule selectivity and instrument sensitivity. Current HRMS systems are reported to have equivalent or better performance than those offered by conventional tandem (MS/MS) quadrupole mass analyzers (15–17). Previous investigators have successfully utilized HRMS such as Q-TOF coupled with LC as a quantitative tool to examine metabolites and define their absorption kinetics in biofluids (18–20). To the best of our knowledge, there is no published HPLC-HRMS method that simultaneously quantifies the provitamin A carotenoids and their bioconversion products in the postprandial plasma triacylglycerol-rich lipoprotein (TRL) fraction.

Thus, the objectives of the present study are to 1) develop an ultra-high sensitive HPLC-HRMS-QTOF for the measurement of the postprandial appearance of provitamin A carotenoids, $\alpha$-retinyl esters and retinyl esters, 2) to determine the vitamin A equivalence of provitamin A-rich biofortified bananas in healthy women.
Dissertation Organization

This dissertation is composed of five chapters, including a general introduction, a literature review, two manuscripts, and a conclusion. The manuscript entitled “High-resolution mass spectrometric analysis for the quantitation of the postprandial plasma appearance of provitamin A carotenoids and their retinyl ester bioconversion products” will be submitted to the *Journal of Agricultural and Food Chemistry*. The manuscript entitled “Vitamin A equivalence of the provitamin A carotenoids in biofortified banana fruit” will be submitted to the *American Journal of Clinical Nutrition*. All the cited literature is based on the format required by the respective journal. This dissertation is concluded with overall results and a discussion of future directions of the research.

References


CHAPTER 2. LITERATURE REVIEW

The essential fat-soluble vitamin A

Fat-soluble vitamin A is in a group of unsaturated organic compounds that are essential to all vertebrates to support physiological function in vision, reproduction, epithelial cell differentiation, and normal growth (1–3). Vitamin A refers to retinoids such as retinol, retinal, retinyl esters, and retinoic acid. Each of these metabolites can be found in the body and must be present in its active forms to support physiological functions. For instance, as an active form of retinal, 11-cis-retinal is needed to bind to the photoreceptor protein (i.e., opsin) for the generation of visual protein rhodopsin; a pigment crucial for a healthy visual cycle (1). The all-trans-retinoic and 9-cis-retinoic acids are transcriptionally active metabolites that bind to the nuclear receptors (e.g., retinoic acid receptor (RAR) and/or retinoid X receptor (RXR)) to regulate specific gene transcription within cells and tissues to support reproduction and embryonic development (2,3).

Humans can obtain dietary vitamin A as a preformed vitamin A and provitamin A carotenoids. An example of the most common dietary preformed vitamin A is retinyl ester, which is a long-chain fatty acid ester of retinol that is richly found in animal origin foods such as dairy products, fish, and meat (4). Provitamin A carotenoid is a precursor of vitamin A richly found in colored fruits and leafy vegetables. Provitamin A carotenoids are isoprenoid compounds with 40 carbon atoms as the backbone with multiple conjugated double bonds and a two-ringed structure (β and/or ε-ionone rings). Figure 1 shows the structures of the selected retinoids and provitamin A carotenoids.
Figure 1. The structures of selected retinoids and provitamin A carotenoids.
Dietary requirements of vitamin A

The dietary requirement of vitamin A is life stage and gender-specific and this requirement can be assessed through a set of reference values known as the dietary reference intakes (DRI). Among the DRI reference values, the Recommended Dietary Allowances (RDA) refers to the average daily dietary intake that is sufficient to meet the nutrition requirement for 97-98% of healthy individuals. The RDA was developed based on the estimated average requirement (EAR), estimated by the dietary vitamin A required to maintain the total vitamin A pool in the body of well-nourished individuals. EAR represents the best-estimated reference value for nutrient intake based on the available experimental and clinical studies and it should be used as the estimate for the daily value of a nutrient (5). In the case where EAR is not available for a specific population (e.g., infants), the adequate intake (AI) is used to estimate the daily requirement.

The National Academy of Medicine (formerly known as Institute of Medicine) has adopted retinol activity equivalents (RAE) as the measurement units for vitamin A requirements. Table 1 summarizes the RDA for vitamin A intake based on gender and life stages. It should be noted that RDA for vitamin A is a reference value to be used as a goal for dietary intake by healthy individuals. RDA should not be used to assess the diets of a population or individuals (5). Based on the estimated RDA, the goal of dietary vitamin A intake for healthy adult men and women over 19 years of age are 900 and 700 μg RAE/day of vitamin A, respectively. For women who are pregnant or lactating, they should achieve a goal of daily vitamin A intake of 750 – 770 to 1,200 – 1,300 RAE. The increasing need for vitamin A in this life stage is required to provide sufficient nutrients for the fetus and infants' growth. As for children at the age of 9 to 13, their need for vitamin A is 600 μg RAE/day. For infants, AI reference value is used for the
estimation of daily intake and this value was based on the average vitamin A intake of infants fed with human milk. The AI for infants aged 0-6 mo and 7-12 mo are 400 and 500 µg RAE/day of vitamin A, respectively.

**Table 1.** Recommended dietary allowance (RDA) for vitamin A intake based on gender and life stage (4)

<table>
<thead>
<tr>
<th>Gender</th>
<th>Adult</th>
<th>Pregnant</th>
<th>Lactation</th>
<th>Children</th>
<th>Adolescent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Men</td>
<td>&gt; 19 y</td>
<td>14-18 y</td>
<td>19-50 y</td>
<td>14-18 y</td>
<td>19-50 y</td>
</tr>
<tr>
<td>Women</td>
<td>900</td>
<td>700</td>
<td>750</td>
<td>770</td>
<td>1,200</td>
</tr>
<tr>
<td>Boys</td>
<td></td>
<td>300</td>
<td>700</td>
<td>700</td>
<td>300</td>
</tr>
<tr>
<td>Girls</td>
<td></td>
<td>300</td>
<td>700</td>
<td>700</td>
<td>300</td>
</tr>
</tbody>
</table>

Other health and lifestyle conditions (e.g., infections, nutrient-nutrient interaction, factors affecting intestinal absorption) can also influence the nutrient requirement (4). For instance, infectious diarrhea resulting from the invasion of enteric pathogens can alter the structure of intestinal mucosa and affect vitamin A absorption (6). The high prevalence of iron and zinc deficiencies can also impact vitamin A metabolism and requirement. Anemia, typically caused by iron deficiency in preschool children in Uganda, is reported as a severe public health significance (7). Iron and marginal vitamin A deficiencies are frequently coexisting and it is supported in the rat model that liver retinol mobilization is impaired during iron deficiency (8). Zinc is an essential cofactor for enzymes involved in vitamin A metabolism. Hence, inadequate zinc in the diet can attenuate enzyme activity (9). Because vitamin A is metabolized similarly to alcohol in the liver, alcohol consumption can negatively influence vitamin A metabolism (10). In addition, iron and zinc have a synergy effect with vitamin A and can influence vitamin A metabolism and increase its requirement.
Recently, the DRI values for vitamin A published in 2001 have been challenged for a revision (11). The rationale of the revision is due to the growing information about vitamin A metabolism such as the advancement of stable isotope methodologies for improved measurement of vitamin A status, a better understanding of retinol and provitamin A carotenoid metabolism, the identified genetic polymorphisms that control the metabolism of β-carotene and the prevalence of single nucleotide polymorphism (SNP) genotypes in human populations.

**Metabolism of vitamin A**

Similar to the fate of cholesterol and dietary lipid intestinal uptake, the digestion of fat-soluble preformed vitamin A (retinyl palmitate) and provitamin A (β-carotene) involves a multistep process that begins with solubilization, packing into mixed micelles and traveling to the brush border membrane for passive diffusion. Solubilization begins with the aids of digestive juice and enzymes such as bile-acids and pancreatic lipases. These juices help to break and emulsify large lipid molecules into small lipid droplets. The small lipid droplets, which are composed of a mixture of phospholipids, cholesterol, and digested lipid products including retinyl palmitate and β-carotene are then packaged into mixed bile salt micelles and diffused to the intestinal brush border membrane for uptake. Hydrolysis of retinyl ester (e.g. retinyl palmitate) to produce free retinol, is an essential step that occurs in the duodenum prior to the uptake by the intestinal cell (12). It is assumed that the duodenum where the pancreatic juice is secreted, contains the enzymes for luminal retinyl ester hydrolysis. Pancreatic lipase is an important key player in this process (13). Retinyl ester can also be hydrolyzed by a hydrolase enzyme found within the intestinal brush border (14).

The intestinal uptake mechanism of free retinol is dose-dependent and involves simple or facilitated diffusion. In an *in vitro* model utilizing the intestinal Caco-2 cell line, free retinol at a
physiological dose (< 150 nM) has been shown to enter the intestinal cells by simple diffusion (15). In contrast, early studies suggested that absorption of free retinol was mediated by a saturable, protein-mediated passive absorption mechanism at a physiological dose and via passive diffusion at pharmacological dosage (450-2700 nM) (16). These observations suggest that the uptake of retinol may involve multiple mechanisms, thus increasing the challenge to identify a single specific protein responsible for the uptake of free retinol in the human enterocytes. However, the potential candidates involved in the process were suggested to be two structurally related receptors, retinol-binding protein-specific receptor Stimulated by Retinoic Acid 6 (STRA 6) (17) and retinol-binding protein-receptor 2 (RBPR2) (18). These receptors exert a specific expression pattern during development and in adults (19) and in the intestine of obese mice (18), respectively. Contrariwise, the uptake mechanism of provitamin A β-carotene is facilitated by a protein-mediated transporter. β-Carotene stays in mixed micelles and is not significantly metabolized or modified. At the duodenum, β-carotene is absorbed intact by class B scavenger integral protein-mediated transporters, known as Scavenger Receptor class B type I (SR-B1) and Cluster Determinant 36 (CD36) (20–22). These transporters are found in numerous tissues especially in the intestine and liver and act as a multifunctional transporter responsible for cholesterol and lipid-soluble vitamin uptake. SR-B1 is shown to be the predominant receptor found in mouse duodenum and it is essential for the uptake of β-carotene when the absorption of β-carotene was compared between wild-type and SR-B1 knockout mice model (22). Its expression is highly regulated by the host vitamin A status via a retinoic acid-induced negative feedback mechanism through the transcription factor Intestine Specific Homeobox (ISX). CD-36, also known as fatty acid transporter, mainly expressed in the jejunum and ileum, has been shown to facilitate β-carotene cellular uptake intestinal (22). Recently, a genotyping of three
candidate gene association studies identified the genetic polymorphisms found in the gene of SR-B1 and CD-36 that may modulate the plasma concentration of provitamin A carotenoid (23,24).

The vitamin A metabolites within the enterocytes are bound to intracellular retinoid transport proteins to be trafficked to their site of action. These include cellular retinol-binding protein type II (CRBP II), cellular retinal binding proteins (CRALBPs), and cellular retinoic acid-binding proteins (CRABPs). Once absorbed, free retinol is bound to CRBP II (25). CRBP II transports retinol to the sites where it is either oxidized to retinal, and then to retinoic acid for gene expression regulation, or esterified to form retinyl esters and incorporate into chylomicron for lymphatic system circulation. Once β-carotene is absorbed into the intestinal cell, it is ready to be cleaved by one of the two cleavage enzymes, cytosolic BCO1 or mitochondria BCO2. A detailed conversion of provitamin A carotenoids within the intestine is discussed in the following section. Some portion of the β-carotene remains intact and bypasses the conversion to vitamin A in the enterocytes, a process highly regulated by individuals’ vitamin A status (26).

The newly absorbed retinyl palmitate and β-carotene, along with the dietary lipids, are packaged into nascent chylomicron within the enterocytes and released first into the lymph and then into general circulation. During circulation, lipoprotein lipase (LPL) catalyzes the hydrolysis of nascent chylomicrons and releases triglycerides and fatty acids for peripheral tissue uptake. The remaining chylomicron and its remnants containing retinyl esters and β-carotene are then traveled to the liver and taken up by the hepatocytes via the low-density lipoprotein (LDL)-surface receptors. It has been shown that the intestine secretes very-low-density lipoproteins (VLDL), a triacylglycerol-rich lipoprotein (TRL) that are packed with more triglycerides and cholesterol, which makes them denser than chylomicrons (27). The postprandial appearance of retinyl esters can be carried by the intestinal VLDL and travel to the liver (28). In the liver,
retinyl esters are released from chylomicron remnants to produce retinol hydrolyzed by retinyl ester hydrolase. Excess retinol can be esterified by lecithin: retinol acyltransferase (LRAT) for storage within the stellate cell (29). The hydrolyzed retinol can be mobilized into plasma as retinol bound to retinol-binding protein-transthyretin complexes (RBP: TTR complex) in which the complexes interact with specific cell receptors on target cells for retinol uptake (30).

**Conversion of provitamin A carotenoid to vitamin A**

The two major sites of the conversion of provitamin A carotenoid to vitamin A are the intestine and liver. The conversion of the absorbed carotenoids into retinol is catalyzed by either one of the two carotenoid cleavage oxygenase enzymes, central cleavage by \( \beta \)-carotene-15,15\(^{\prime} \) oxygenase (BCO1) (31,32) or eccentric cleavage by \( \beta \)-carotene-9\(^{\prime},10^{\prime} \)-oxygenase (BCO2) (33). BCO1 and BCO2 belong to a superfamily of non-heme iron-dependent oxygenase. The cellular compartmentalization, substrate specificity, and production of various products to support physiological functions reflect the distinct role of each of these enzymes in carotenoid metabolisms within the body.

BCO1 and BCO2 show distinct differences in their cell-type-specific expression (34,35), subcellular localization (36), preferred cleavage substrates, and selective location for cleavage reaction (37). BCO1 is a cytosolic enzyme highly expressed in the intestine, and its preferred substrates are provitamin A carotenoids. In contrast, BCO2 is a mitochondrial enzyme residing in the inner membrane of the mitochondrial that expresses mainly in the hepatocytes (38). Studies have shown that BCO2 has a broad substrate specificity; it cleaves specific isomers of lycopene (39) and lutein (40). BCO2 in mitochondria helps to prevent oxidative stress caused by excessive accumulation of carotenoids (38).
BCO1 conducts symmetrical cleavage of provitamin A carotenoid to vitamin A in the intestine when the vitamin A supply is low. Central cleavage of β-carotene at 15, 15’ bond by BCO1 can produce two molecules of all-trans-retinal (32). The retinal formed from β-carotene cleavage will oxidize to form retinoic acids or reduce to retinol and esterify to form retinyl esters depending on the host vitamin A needs. It is well recognized that the conversion of β-carotene to vitamin A is highly variable and this phenomenon has recently been explained by the identification of two common nonsynonymous single nucleotide polymorphisms (SNPs) within the BCO1 coding region (41). An SNP is the difference of a nucleotide base pair at a single position of the DNA sequence among individuals, where the occurrence of this variation is > 1% within a population (42). Individuals with low capacity to form vitamin A from β-carotene were classified as poor converters. These participants showed a high circulating plasma β-carotene and low retinyl palmitate: β-carotene ratio after the ingestion of a supplemented β-carotene dose. Poor converters were shown to have the two genetic polymorphisms (SNPs) on the BCO1 coding region with reducing catalytic activity of BCO1 (41).

**α-Retinol and α-retinyl esters**

BCO1 cleaves nonsymmetrical provitamin A α-carotene at 15,15’ double bond to produce one molecule of α-retinal (non-vitamin A product) and retinal (vitamin A), and reduction of these molecules produces α-retinol and retinol. α-Retinal and retinal are different in a double bond position; an ε-ionone ring (double bond on the 4,5-position of retinol) versus a β-ionone ring (double bond on the 5,6-position of retinol) (Figure 2). Within the enterocyte, α-retinol can be esterified to α-retinyl ester and packaged into chylomicron and traveled to the liver. The earlier investigations reported a discrepancy in vitamin A activity of α-retinol and retinol using *in vitro* and *in vivo* models (43). α-Retinol was shown in a rat growth bioassay test
with limited growth activity (44). However, in a separate study where the α-retinyl acetate was delivered as a source of α-retinol directly to hamster tracheas harvested as the in vitro media, α-retinol was shown to replace retinol in maintaining normal cell differentiation (45). When α-carotene was fed to the weaning rat, α-metabolites accumulated in the liver extract (46). A subsequent study showed that α-retinol has a low binding affinity to retinol-binding protein (RBP) hence inhibiting its ability to circulate as α-retinol-bound RBP (47). The cellular uptake of retinol requires RBP-bound retinol (holo-RBP) to act as a ligand to a specific membrane receptor; α-retinol without RBP is assumed to have limited vitamin A activity. A study published recently confirmed that α-retinol travels to other tissues in its esterified form and utilizes locally as α-retinol in vitamin A-deficient piglet fed with α-retinyl acetate (48). The metabolism of α-retinol in animals has become clear, that α-retinol can act as retinol within a cell, but the low bioactivity of α-retinol shown in growth-promoting bioassays has been attributed to the absence of a transportation mechanism. These reports supported the importance of understanding the α-metabolites metabolism, where α-retinol can be esterified, transported via chylomicrons, and stored in the liver and recirculated into the body in its esterified form. However, there is a lack of information regarding α-retinol metabolism and its vitamin A activity in humans.
Regulation of vitamin A in the body

Vitamin A homeostasis refers to the constant maintenance of vitamin A metabolites in the body that is necessary to support normal physiological functions. This homeostasis process is tightly regulated by the production of retinol within the body, and the major sites of regulations discussed here focused on the regulations in the intestine and in the liver. Retinol can be produced from the ingested preformed vitamin A that is absorbed with up to 99% efficiency \(^\text{(49)}\). However, the production of retinol from preformed vitamin A is not regulated within the body, thus, excessive intake of preformed vitamin A can cause hypervitaminosis A, a condition caused by vitamin A toxicity \(^\text{(50)}\). In contrast, retinol produced from the ingested provitamin A carotenoid is regulated depending on the host’s vitamin A status. Total body vitamin A status regulates the intestinal absorption and conversion of provitamin A carotenoid as well as the hepatic esterification and excretion processes through regulating the expression of the 1) intestinal membrane receptor SR-B1, 2) cytosolic cleavage enzyme BCO1, 3) esterification enzyme lecithin: retinol acyltransferase (LRAT), and 4) clearance protein cytochrome P450 family 26 enzymes (CYP 26).
In the intestine, the expression of SR-B1 and BCO1 is regulated through a diet-responsive regulatory network via an intestinal transcription factor Intestine Specific Homeobox (ISX) (51–53). Through a feedback mechanism, the excess cellular retinoic acid binds to the retinoic acid receptor (RAR), or retinoic X receptor (RXR) which increases the expression of ISX. The expression of the ISX transcription factor suppresses the gene that regulates SR-B1 and BCO1 protein expressions resulting in the reduction in the catalytic activity of SR-B1 and BCO1. Hence, this reduces the absorption of provitamin A and its conversion to vitamin A. However, the provitamin A conversion efficiency among individuals is highly variable and this may be attributed to the contribution of genetic polymorphism variation. Recently, several single nucleotide polymorphisms (SNPs) have been identified to be associated with the gene that encodes for BCO1 protein (41,54).

Vitamin A homeostasis can be achieved through hepatic retinol metabolism (55). The hepatic vitamin A (retinol) level can be regulated through the expression of hepatic enzymes such as lecithin: retinol acyltransferase (LRAT) and cytochrome P450, also known as CYP26 (55). Hepatic LRAT, a microsomal protein that catalyzes the esterification of CRBP II-bound free retinol to retinyl esters in the intestine and liver, was previously shown to be directly affected by vitamin A status in rats; the undetectable hepatic LRAT activity is observed in vitamin A-deficient rats (56). A subsequent study concluded the role of retinoic acid in regulating LRAT activity; the LRAT activity was restored in vitamin A-deficient rats fed with retinoic acid (57). As the active metabolite of retinol, retinoic acid is essential in the regulation of hepatic LRAT expression. The catabolism of retinoic acid involves CYP26 enzymes in the liver where excess retinoic acid is eliminated by CYP26 through the production of polar retinoic acid metabolites. Excess retinoic acid binds to the nuclear receptor and increases the gene
transcription and the production of CYP26 enzyme to increase the clearance of retinoic acid within the cell (58). Vitamin A status of an individual can affect the expression of CYP26, which in turn affects vitamin A metabolism. Although the overall knowledge of the hepatic regulation of retinol has been obtained from these studies, more evidence is needed to find the association of vitamin A status and retinoic acid clearance. Another possible contribution to the retinoic acid clearance mechanism involves the genetic variants identified in CYP26 (59).

**Vitamin A deficiency as a public health concern**

Vitamin A deficiency in children remains prevalent in South Asia and sub-Saharan Africa (60). Vitamin A deficiency is a public health concern defined by low serum retinol level (< 0.70 μmol/L) or depleted liver vitamin A storage (< 0.07 μmol/g) (61). The earliest onset of vitamin A deficiency in preschool-aged children results in an increased risk of infections. High risk of infection rates due to poor immunity increases their vulnerability to measles and diarrheal infections, which are the leading causes of death before the aged of 5 (62). Vitamin A deficiency is also prevalent in women of reproductive age (63). Pregnant women with low serum retinol are prone to gestational night blindness during pregnancy where the condition may worsen the health of the mother and the newborn (64–66). Untreated night blindness has led to the development of a spectrum of ocular diseases known as xerophthalmia (67). Hence, the global initiatives to improve vitamin A status in children focused on reducing childhood mortality as well as preventing night blindness.

**Vitamin A deficiency in Uganda**

Uganda located in East-central Africa is among the African countries at risk with vitamin A deficiency. More than one-third of the Ugandan preschool children and women of reproductive age are vitamin A deficient (68). The monotonous and unvaried diets low in
micronutrients (e.g. plantain, cassava and sweet potatoes) (69) consumed by Ugandans contribute to the risk of vitamin A deficiency. As one of the important cash crop staples, Ugandans consume more than 200 kg of banana per year (including non-Cavendish and plantains) (70). In general, bananas, which include plantains, grown in Uganda are rich in starch, and essential minerals potassium, calcium, phosphorus, and magnesium but contain very low levels of fats and proteins (71). As a common weaning food, Ugandan infants and young children consume traditional banana-based starchy staple “Matooke” that is low in fat-soluble vitamin A. Hence, it became evident that the Ugandan diet relies heavily on a single staple and lacks dietary diversification as a risk factor for early childhood malnutrition including vitamin A deficiency in infants and young children under 30 month-old (72,73).

Although high-dose vitamin A supplementation and commodity fortifications (e.g., sugar, oil and fats) have been introduced to reduce vitamin A deficiency, there is still limited delivery coverage of vitamin A supplementation on serum retinol and ineffective food fortification programs reported (69,74). According to the UNICEF 2018 vitamin A supplementation report, the coverage rates for two-dose vitamin A supplementation was only 33%, indicating more than two-third of the total population at 6 to 59-months did not receive supplementation (75). In fact, vitamin A supplementation only shows a transient effect on serum retinol of the vulnerable (76). The limited responsiveness of serum retinol to the supplementation suggested the need for a sustainable approach to eliminate vitamin A deficiency.

Because the potential impacts of commodity fortifications on household members rely heavily on the food consumption patterns and nutrient requirements, this approach could be effective if only the formulation adjustment is completed through the continuous assessment of dietary intake (77). However, dietary assessment is often challenging and time-consuming. As a
main commercial form food fortificant, preformed vitamin A is not a safe dietary vitamin A source (50,77). A simulation model was used to assess the impact of fortification in vegetable oil and sugar and concluded that food-based fortification may eliminate vitamin A deficiency with a risk of 2%-48% children exceeding the tolerable upper intake level (77). To alleviate the spread of vitamin A and other micronutrient deficiencies, a safe and cost-effective approach is needed as complementary to the existing interventions.

**Provitamin A carotenoids as vitamin A sources**

Provitamin A carotenoids in the plant-based diet account for more than 80% of dietary vitamin A in many African countries as a major source of dietary energy (78). As a major source of dietary energy, these plant-based diets are often starchy staples low in micronutrients. Only 11-13% of the dietary energy is supplied through meat products (74). Because plant-based and starchy staples are widely consumed in these regions, improving the provitamin A quality of these foods can increase vitamin A intake to meet the adequate daily requirement of the vitamin A deficiency of at-risk populations with the potential to end the endemic.

Biofortification is a technology to improve micronutrients in a crop through plant breeding. Through conventional or transgenic breeding techniques, the density of micronutrients in staple crops such as provitamin A carotenoids can be increased to the extent to improve vitamin A status of the vulnerable populations. Examples of provitamin A carotenoid enriched biofortified crops that are available in several African countries are orange maize, yellow cassava, and orange-fleshed sweet potato (79). Based on the published bioavailability and bioefficacy studies, these crops have demonstrated their effectiveness in alleviating vitamin A deficiency. In a controlled human study, 6.48 μg of β-carotene biofortified maize was shown to be as bioavailable as 1 μg of retinol (80). The bioefficacy of β-carotene biofortified maize has
also successfully demonstrated in maintaining the vitamin A status of Mongolian gerbil and healthy men in Zimbabwe (81,82). Similarly, liver vitamin A reserves were elevated when children were fed with enhanced vitamin A level biofortified orange maize (83). It was also reported that 4.2 μg of β-carotene in biofortified yellow cassava was as bioavailable as 1 μg of retinol in healthy women (84) and a bioefficacy study in Kenya showed that biofortified yellow cassava improved the serum retinol of Kenya school children (85). A bioefficacy study showed that orange-fleshed sweet potato enriched with provitamin A carotenoids significantly increased in vitamin A body reserve in young children in Mozambique (86). Transgenic plant breeding allows the introduction of provitamin A that does not naturally exist in a single staple crop (e.g., rice). Through the transgenic technique, the new generation of “golden rice 2” was designed to provide a maximum of 37 μg/g β-carotene (87). This golden rice with improved provitamin A carotenoid shows a bioavailability conversion factor of 3.8 μg to 1 μg of retinol in healthy adults (88). However, the transgenic crop has not been made available for adoption due to foreign policy in adopting genetically modified organisms (89).

**Provitamin A enriched-transgenic bananas**

The domestication of bananas allows the selection of favorable traits including transforming a full of seeds inedible wild banana to seedless edible domesticated bananas. As a result, domestication leads to near-sterile triploids and seedless cultivars; thus, crop improvement through conventional breeding becomes challenging (90). Due to low fertility, genetic modification is the only possible way for crop improvement in most triploid domesticated bananas (91). Domestication also produces a wide variety of cultivars, including dessert, cooking, roasting, and beer bananas found in Africa (92), and also cultivars with high-level of provitamin A carotenoids found in Papua New Guinea (93,94). Genetic modification has
allowed the specific gene isolated from the high-provitamin A accumulating cultivar to be transferred to the low-provitamin A accumulating cultivar to produce a culturally acceptable provitamin A carotenoid-rich banana cultivar that is widely consumed by East-central Africans.

As a proof-of-concept, the “Cavendish” cultivar was first chosen as the model to test the genetic modification technique and identify the appropriate promoter and cis/transgene for stable expression of the gene that generates high provitamin A level (90,95,96). To meet 50% of the EAR of vitamin A in the vulnerable populations, plant scientists must achieve a target value of 20 µg/g β-carotene equivalent (β-CE) in the banana fresh fruit (97). The leading experts from the Queensland University of Technology in Australia and the National Agricultural Research Organization (NARO) of Uganda continue to improve the provitamin A content of the fruit (98).

**Vitamin A activity of provitamin A carotenoids**

Vitamin A activity of provitamin A carotenoids in foods can be expressed as retinol activity equivalent (RAE) – i.e., the quantity of the provitamin A carotenoid expressed as β-carotene equivalents needed to provide vitamin A activity equivalent to 1 µg of retinol (4). RAE is a conversion unit developed to replace the international unit Retinol Equivalent (RE). RAE was developed based on the existing absorption studies on the effect of food matrix or other factors on bioaccessibility and bioavailability of provitamin A carotenoids. The National Academy of Medicine has concluded that 1 µg preformed vitamin A (retinol), 2 µg of supplemented β-carotene in oil and 12 µg β-carotene, 24 µg α-carotene and β-cryptoxanthin from foods are required respectively to provide 1 µg RAE of retinol (4). Table 2 summarizes the RAE conversion ratio for the provitamin A carotenoids.
Table 2. Comparison of retinol activity equivalent (RAE) and retinol equivalent (RE) conversion factors (4)

<table>
<thead>
<tr>
<th></th>
<th>Retinol Activity Equivalent (RAE)</th>
<th>Retinol Equivalent (RE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 µg RAE</td>
<td>1 µg RE</td>
</tr>
<tr>
<td>All-trans-retinol</td>
<td>1 µg</td>
<td>1 µg</td>
</tr>
<tr>
<td>Supplemental all-trans-β-carotene</td>
<td>2 µg</td>
<td>2 µg</td>
</tr>
<tr>
<td>Dietary all-trans-β-carotene</td>
<td>12 µg</td>
<td>6 µg</td>
</tr>
<tr>
<td>Dietary α-carotene or β-cryptoxanthin</td>
<td>24 µg</td>
<td>12 µg</td>
</tr>
</tbody>
</table>

Measuring vitamin A activity of provitamin A carotenoids in humans

The absorption efficiency of provitamin A carotenoids in humans has been widely studied using various techniques (99). These techniques seek to measure the bioconversion of dietary provitamin A carotenoids to vitamin A expressed as vitamin A equivalents or retinol activity equivalents. Bioconversion, which refers to the fraction of bioavailable provitamin A that can be converted into retinol (100), is greatly influenced by the proportion of an ingested carotenoid that can be utilized for normal physiological function or storage (i.e. bioavailability) (100). The bioavailability is impacted by the proportion of a carotenoid that is transferred from the food to micelles during digestion and made accessible for intestinal absorption (i.e. bioaccessibility) (101). The bioavailability of provitamin A carotenoid in plants can also be influenced by the food matrices where carotenoids are embedded as well as other factors such as host vitamin A status or the presence of dietary fat.

The study of bioconversion of β-carotene or other provitamin A carotenoids to retinol in humans is challenging. To understand the postprandial absorption kinetics and absorption capacity of the provitamin A carotenoids at a physiological dose, the experimental method must have the ability to differentiate the newly absorbed carotenoids and the newly formed vitamin A
from the body reserves, which greatly correlate with the vitamin A status of the individuals. These methods must have the ability to quantitatively assess the bioefficacy without posing any potential health threat (e.g., depletion-repletion and radioisotope-labeled techniques have been banned for use). Hence, several methods have been developed to overcome this challenge and applied to populations with different vitamin A status (e.g., marginal vitamin A status, adequate vitamin A status). Herein, the discussion will only focus on methods that quantify the vitamin A bioconversion factor of provitamin A carotenoid in humans that are relevant to the present study. These assessments include stable isotope reference method (IRM) and postprandial triacylglycerol-rich lipoprotein fraction response curve. Both methods involve the administration of a known amount of vitamin A reference dose to quantify the vitamin A equivalents of provitamin A carotenoids in foods.

**Stable-isotope reference method (IRM)**

The stable-isotope reference method utilizes the intrinsic mineral labeling of plants and a known amount of labeled reference dose to quantify the bioconversion of provitamin A carotenoid to retinol. The isotope labeling in plants allowed the differentiation of the endogenous retinol with the newly formed labeled [²H] retinol. Generally, IRM is considered safe when chemically pure and stable tracers at the appropriate dosage are used (102). The formation of [¹³C] retinol from the administration of a known amount of ¹³C₁₀-retinyl acetate reference dose and [²H] retinol from the isotopically labeled plant material allows the comparison of post-absorptive bioconversion of provitamin A carotenoids in the liver and other tissues to retinol. By assessing the amount of retinol formed from the administration of a known amount of labeled ¹³C₁₀-retinyl acetate reference dose, a comparison can be made to determine the RAE of provitamin A carotenoid of the ²H₈ β-carotene-labeled plant and the measured retinol
formed after consumption of the plant material. The retinol formed can be measured using the area under the curve (AUC), which represents the fractions of retinol absorbed in the serum across time. IRM was applied to assess the bioconversion of deuterated spinach and carrots (103), deuterated spirulina (104), and deuterated β-carotene enriched golden rice (88) in populations with serum vitamin A level ranging from marginal to normal (Table 3). These plant materials were intrinsically labeled with a low abundance of stable isotopes to produce isotopic tag elements that can be distinguished from the natural form of the element (e.g., $^{13}$C and $^{12}$C, or $^2$H and $^1$H).

**Table 3.** Vitamin A bioconversion factor of deuterated provitamin A carotenoid-rich-plants measured by stable isotope reference method (IRM)

<table>
<thead>
<tr>
<th>Intrinsically labeled foods</th>
<th>Vitamin A equivalency</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinach</td>
<td>20.9</td>
<td>Tang et al. (171)</td>
</tr>
<tr>
<td>Carrot</td>
<td>14.8</td>
<td></td>
</tr>
<tr>
<td>Spirulina</td>
<td>4.5</td>
<td>Wang et al. (111)</td>
</tr>
<tr>
<td>Golden rice</td>
<td>3.8</td>
<td>Tang et al. (88)</td>
</tr>
</tbody>
</table>

**Postprandial triacylglycerol-rich lipoprotein (TRL) response curve method**

Our laboratory developed a postprandial TRL response curve method to measure the RAE of the β-carotene biofortified maize (80). This method requires the consumption of a provitamin A carotenoid-rich food, and a known amount of vitamin A reference dose served in a control matrix. Similar to the approach used in IRM, the use of a known amount of reference dose in this method allows the determination of the RAE of the provitamin A-rich crop where the AUC produced after the consumption of provitamin A-rich food is compared with that of the vitamin A reference dose. Contrary to IRM, where post-absorptive bioconversion can be
measured in the liver and other tissues, the postprandial TRL method only measures the intestinal conversion efficiency of provitamin A carotenoids to retinol.

The postprandial response of provitamin A carotenoid such as β-carotene can only be evaluated in the appropriate plasma lipoprotein fractions in order to distinguish its origin from the endogenous concentration; carotenoids have a long half-life in circulation (105). Several reports have investigated which fractions within the plasma are best to represent the intestinal absorption and bioconversion of β-carotene to retinol. The use of total plasma TRL fractions as the vehicle to study the appearance of β-carotene and retinyl esters derived from β-carotene cleavage in the intestine is based on the assumption that total plasma TRL fractions are composed primarily of intestinal origin lipoproteins which include chylomicrons and its remnants, as well as some VLDLs upon absorption (106). However, when the total plasma TRL fractions were isolated and their apolipoprotein compositions were measured after the ingestion of dietary fat, the increase of the hepatic origin apolipoprotein-containing VLDLs (apoB-100) exceed the intestinal origin apolipoprotein-containing chylomicrons (apoB-48) (107). The apoB-100 in total plasma TRL has been attributed to the hepatic contribution of VLDLs as a result of saturation of lipoprotein lipase activity by chylomicrons (108). Hence utilizing total plasma TRL which includes the hepatic origin β-carotene in VLDLs may overestimate the intestinal β-carotene absorption. However, earlier investigations have indicated the maximum appearance of oral ingested β-carotene was found in both chylomicron and VLDL fractions at 5-6 hours after ingestion (109,110). These observations have led to the interest in characterizing the VLDL subfraction in order to better distinguish the exogenous and endogenous origin of β-carotene. Our research group has previously fractionated the TRL to characterize their utility for the postprandial β-carotene appearance by their particle size and composition (111,112). The
authors observed that the β-carotene appearance in the VLDL\textsubscript{A} at 6 h exceeded that in chylomicrons, but not in other subfractions of VLDL. The rapid accumulation of β-carotene in the VLDL\textsubscript{A} might represent contamination with chylomicron remnants. This rapid accumulation of β-carotene in VLDL\textsubscript{A} was reported previously (109,110).

The liver is the major storage site for vitamin A (retinyl esters) in healthy individuals. The excretion of retinyl esters from the liver after a vitamin A-rich meal reflects only the intestinal absorption (113) and can be used as a great postprandial indicator of intestinally derived TRL. Our laboratory utilized the chylomicrons as well as VLDL\textsubscript{A} to capture the postprandial appearance of retinyl esters. Estimating the postprandial appearance of retinyl palmitate in the chylomicron and its remnants alone has been suggested to underestimate the total vitamin A absorption because retinyl esters formed in the intestine can also circulate in the intestinal-origin VLDL (28). Other studies from our laboratory previously observed that the postprandial appearance of retinyl palmitate in VLDL\textsubscript{A} corresponds with that of chylomicron in time and magnitude (112). This observation suggested that the postprandial appearance of retinyl esters in large VLDL can reflect their appearance in chylomicron remnants (114). Retinyl palmitate was measured as the dominant retinyl ester because the postprandial relative distribution of retinyl esters is virtually constant during absorption (115). The use of the TRL response curve to study the bioconversion of provitamin A can only be adopted by a healthy population with normal vitamin A status where high conversion efficiency is expected in the intestine. With the use of chylomicrons and VLDL\textsubscript{A} as the vehicles to study the bioconversion of biofortified maize, Li et al. concluded that 6.48 μg of the unlabeled dosage β-carotene enriched biofortified maize is required to provide the vitamin A activity equivalent to 1 μg retinol (80). Our laboratory validated the accuracy of the overall study design using a known
amount of β-carotene reference dose (80). The vitamin A equivalence of β-carotene in the reference dose was determined to be 2.34:1, which is in agreement with the value reported by the National Academy of Medicine (4). This study highlighted the use of postprandial dose-response curves using TRL fractions, specifically chylomicron and VLDL_A, as an accurate assessment of the intestinal absorption and conversion of β-carotene to its bioconversion products in provitamin A-rich foods.

**Extraction and analysis of vitamin A metabolites in biological samples**

**Lipoprotein separation and extraction**

After the ingestion of a fat-rich meal, fat-soluble vitamin A circulates in the body in the form of lipoproteins, thus marking the importance of the separation of plasma lipoprotein fractions for postprandial vitamin A extraction. Cumulative rate-ultracentrifugation is considered the “gold standard” for the isolation of triacylglycerol-rich lipoproteins. It has been reported that the validity of using cumulative-density ultracentrifugation to quantify postprandial TRL is supported by another comparable technique using nuclear magnetic resonance (NMR) spectrometry (116). Cumulative rate-ultracentrifugation separates the fractions based on the lipoprotein floatation density index (Sf) and particle shape and size with high purity and recovery rates (116). The use of various salt gradient densities (e.g., sodium chloride and potassium bromide) and ultracentrifugation can progressively sub-fractionate the fractions into chylomicron, VLDL_A, VLDL_B, and VLDL_C (111). As each fraction decreases in diameter, it increases in density. To confirm the purity of each extracted lipoprotein fraction as indicated by the flotation density (Sf), the specific apolipoprotein that is distinctive to its fraction can be identified using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). SDS-PAGE separates the apolipoproteins based on their molecular masses from different polypeptide
chain length. For instance, apoB-100 is a hepatic origin apolipoprotein that is characterized by $S_f$ 20-400, it is unique to the VLDL sub-fractions (114) and have a molecular mass of 540,000 Da. In contrast, apoB-48 is synthesized exclusively in the small intestine, it is characterized by $S_f > 400$ that is unique to chylomicron and its remnants. It has a molecular mass of 48% of that of apoB-100. Hence, quantifying the apoB-48 and apoB-100 in the particles with $S_f < 1000$ may provide information on the origin of retinyl esters and provitamin A in the plasma.

Paetau and others characterized the triacylglycerol-rich lipoproteins based on their $S_f$ index and appearance of apoB in each fraction (111). The authors subfractionated the VLDL into VLDL$_A$, VLDL$_B$ and VLDL$_C$ and observed that within the sub-fractions of VLDL$_s$, β-carotene was richly found mainly in VLDL$_A$ ($S_f$ 175-400) after the ingestion of β-carotene dose. This observation indicates that the appearance of β-carotene in the VLDL$_A$ fraction could contribute to the postdosing effect of absorbed β-carotene; chylomicron remnants contamination in VLDL$_A$. However, there were no intestinal origin apoB-48 detected in the VLDL$_A$.

These fractions can be subjected to HPLC analysis to accurately quantify postprandial provitamin A carotenoid bioavailability in humans. This extraction method has been adopted by various investigators to study the bioconversion of provitamin A carotenoid in humans (80,117). Thus, for accurate assessment of the postprandial bioconversion factor of provitamin A carotenoid to vitamin A after the consumption of provitamin A rich foods, a high confidence lipoprotein fractionation method is required to measure β-carotene absorption and its vitamin A capacity.

**Vitamin A analysis using reversed-phase high-performance liquid chromatography**

Reversed-phase high-performance liquid chromatography (RP-HPLC) is a common analytical chromatography tool for the analysis of retinoids and carotenoids. RP-HPLC uses columns (e.g., C18 and C30) that are filled with modified non-polar silica particles as the
stationary phase, and a more polar mobile phase for separation. Many published RP-HPLC methods use isocratic or gradient mode on a C18 column for retinoid and carotenoid separation (118,119). Previously, a C18 column was used to separate \( \alpha \)-retinol and retinol after samples were subjected to saponification to identify \( \alpha \)-retinyl and retinyl esters (120). In general, C18 columns are more prone to retention time shifts and have poor selectivity for geometrical isomers of carotenoid and polar carotenoids (e.g., lutein and zeaxanthin) (118). However, the introduction of the C30 Carotenoid Column in recent years has provided a lower tendency for retention time shift and greater selectivity for cis-trans carotenoid isomers (121,122). The C30 Carotenoid Column is capable of resolving multiple fat-soluble analytes and the selected isomers such as retinoids, tocopherols, the stereoisomer of tocopherols, and carotenoids (117). C30 Carotenoid Column has been utilized in RP-HPLC for the analysis of retinoids and carotenoids in biological matrices (80,117,123,124). However, the commercially available C30 Carotenoid Column offers only particle sizes and column diameter that allows lengthy analysis time, which is one of the major drawbacks of utilizing C30 Carotenoid Column for RP-HPLC analysis. When 3 \( \mu \)m particle size was used, the C30 carotenoid column reduced the separation time to 35 mins (124). It has been demonstrated previously that the C30 carotenoid column can resolve \( \alpha \)-retinyl esters from retinyl esters where the molecule identities were validated on mass spectrometry (125).

**Detector**

RPLC systems are equipped with a detection unit for identification and quantitation purposes. Due to the low biological levels of retinoids and carotenoids, a highly sensitive robust detector is required for postprandial measurement of bioconversion products contributed by provitamin A carotenoids. HPLC is commonly coupled with detectors including the photodiode-
array detector (PDA), electrochemical detection (ECD), and mass spectrometry (MS), which have been used to quantify retinoids and carotenoids in biological samples. These detectors vary in terms of their cost of operation, ease of use, sensitivity, and robustness.

PDA detector is widely used to analyze molecules with absorbance properties such as vitamin A. The presence of chromophores (i.e., conjugated double-bond system) in retinoids and carotenoids provide several unique absorbance properties in a specific region in the ultra-violet (UV) light spectrum. These absorbance properties obey Beer-Lambert’s law as their principle of detection, where the absorbance response is linearly correlated with the sample concentration. The cost-effective and highly robust HPLC-PDA has been used in the determination of retinoids in serum and tissues (123,126), total carotenoids in humans (122), and serum concentration of numerous retinoids and carotenoids (127). PDA detector can be limited to analysis involving analytes with overlapping wavelengths, affecting its accuracy in quantitation. This detector may not have the detection limit for the detection of the postprandial appearance of carotenoids and vitamin A in the biological samples.

Electrochemical detection (ECD) provides greater sensitivity as compared with UV detection to detect neurotransmitters and endogenous vitamin A in biological samples, especially when the amount of the sample is limited. The measurement of the electrical current of the electrochemically active compounds (i.e., carotenoids, tocopherols, and retinoids) in biological samples has been demonstrated in various studies (80,117,128–130). The detector measures the oxidation or reduction reaction of the electrochemically active analytes when a voltage potential is applied. The concentration of an analyte is directly proportional to the voltage generated, as reflected in the analyte’s electrochemical reaction and the production of the electric current. It was reported that the detection limits for β-carotene measured by ECD are 100- to 1000-fold
enhanced as compared with the UV-Vis detectors (130). Although the ECD technique has adequate sensitivity to measure most of the vitamin A present in the biological samples (80,84,129), some electrochemically inactive compounds, such as $\alpha$-retinyl esters, may not be detected.

**Measuring provitamin A carotenoid and their bioconversion products using LC-MS**

High-performance liquid-chromatography-mass spectrometry (HPLC-MS) is a widely used analytical technique in stable isotope dilution studies where the ingestion of stable isotopic tracer labeled-foods captured the vitamin A absorption kinetic to study provitamin A carotenoid bioavailability, bioconversion, and/or vitamin A status in humans (103,131–134). Without the ingestion of stable isotopic tracer, HPLC-MS has demonstrated sufficient sensitivity to detect the postprandial appearance of provitamin A carotenoids and their bioconversion products in humans (135,136). Previously, Kopec and others developed a method using HPLC-PDA interfaced with a quadrupole-TRAP (Q-TRAP) mass spectrometer operated tandem in series (HPLC-MS/MS) to detect the analytes based on the specific precursor-product transitions (136,137). Goetz and others developed an LC-MS/MS method to achieve the molecule selectivity to differentiate $\alpha$-retinyl esters from retinyl esters by monitoring the corresponding transitions (125). This method was applied to a subsequent study for postprandial detection and quantitation of provitamin A carotenoids and their bioconversion products after the ingestion of $\alpha$-carotene containing carrot meal (135). These studies have highlighted the use of a traditional HPLC-tandem MS (HPLC-MS/MS) monitored through selected reaction monitoring (SRM) acquisition mode for quantitative analysis in provitamin A carotenoid absorption studies.
Liquid-chromatography tandem mass spectrometry (LC-MS/MS)

For several decades, HPLC-MS/MS method has been the most employed instrumentation technique for quantitative analysis due to its selectivity offered by SRM acquisition mode. SRM is a quantitative target analyte scan; it increases the molecule selectivity by identifying the daughter ion produced by a specified precursor ion fragmented under the defined MS condition. SRM acquisition scan mode maximizes the assay sensitivity by monitoring the preselected number of analytes for quantitation; it is suitable for targeted analysis in a well-developed quantitative assay. However, SRM scanning mode is not commonly used for non-targeted metabolite screening applications, especially in the presence of unknown matrices (138). SRM has limited ability to differentiate isomers with similar elemental formula or unknown species with the same nominal molecular mass as the target analyte which can be detrimental to the validity of the analysis (138). The interference of isobaric mass transitions and isomers present in complex biological samples may reduce the selectivity of MS/MS. Although MS/MS coupling with LC may provide chromatographic separation and increase the selectivity, some compounds may not resolve under the simple chromatography condition, which leads to the need for intensive method development. Implementation of a new LC-MS/MS assay requires extensive MS experience and can be time-consuming (139). To understand how a specific ion is fragmented under the MS condition in the presence of an unknown matrix background, the laboratory personnel must possess in-depth knowledge in biochemistry and chemistry. To assure assay accuracy, verification of the assays is required to confirm the identity of the selected fragmented ion of the target analytes. Also, HPLC-MS/MS lacks the ability to provide qualitative analysis such as structural elucidation and compound identification. Recently, there is growing interest in shifting from LC-MS/MS to LC coupled with high-resolution mass
spectrometry (LC-HRMS), hence switching the acquisition mode from SRM to full scan HRMS analysis (140).

**High-resolution mass spectrometry (HRMS)**

High-resolution mass spectrometry (HRMS), such as Orbitrap, quadrupole time-of-flight (Q-TOF), has evolved to be the top analytical tool for qualitative and quantitative analysis in bioanalysis (141–143). The high resolving power offered by HRMS allows the exact mass measurement of the targeted molecules in the presence of isobaric compounds within the matrices and this trait is a desirable feature for drug discovery in complex biological matrices (e.g., human plasma). HRMS such as Q-TOF offers full-scan acquisition mode that allows retrospective analysis within a single injection (142). Full-scan acquisition resulted in a full-mass range plot known as the total ion current plot, where all ions in each MS scan could be plotted against the intensity. The collection of all ions in one single plot allows qualitative analysis such as elucidating the structures of the unknown compounds for metabolic profiling. The new generation of HRMS with improvement made in ion source selection or time-to-digital converter has shown improved linear dynamic range that was once suffered by the previous generation of Q-TOF (143). In principle, Q-TOF utilizes a front-end quadrupole filter to isolate the precursor ion, followed by a flight tube device (time-of-flight) that measures the mass-to-charge (m/z) ratio of all the accelerated charged ions based on the kinetic energy and the velocity of the ions. With a constant applied voltage, charges with different molecular mass can be separated by the flight time.

The benefits of HRMS has led us to explore the use of HPLC-HRMS-QTOF for the quantitation of postprandial provitamin A and retinyl ester bioconversion products in triacylglycerol-rich lipoprotein fractions. We believe this instrumentation will have the selectivity and sensitivity needed to separate, identify, and quantify α-carotene, β-carotene, α-
retinyl esters and retinyl esters in postprandial TRL fractions. The present study demonstrates the ultra-high selectivity and sensitivity of HPLC-HRMS-QTOF as a quantitative analytical tool in measuring postprandial responses of provitamin A carotenoids and their bioconversion products after the consumption of α-carotene containing foods.

**Ionization techniques**

Electrospray ionization (ESI) is the most widely used atmospheric pressure ionization technique for LC-MS application due to its broad applicability for intact and large biomolecules like proteins and peptides (144–146). Typically, samples are subjected to an analytical column for separation and then introduced to the ion source for ionization to generate gaseous charged ions before the charged ions are sent to the mass analyzer for mass differentiation by their specific mass-to-charge ratio (m/z). As the name implies, the electrospray mechanism involves the simultaneous occurrence of droplet formation and charging (147). The eluent is dispersed into a fine mist of charged droplets as it passes through the nebulizer at a high temperature with a constant flow of dry nitrogen and a highly charged capillary at a specific voltage. These charged droplets travel to the mass analyzer to be sorted based on their m/z ratio.

The electrospray technique has been demonstrated in LC-MS analysis involving retinoids (148) and carotenoids (149). It has been suggested that the ionization of retinoids is specific to the electrospray condition and some retinoids (e.g., retinoic acid, retinol, or retinal) ionize more efficiently in negative ion mode (148). Also, it has been concluded that retinoids and carotenoids exert different ionization mechanisms (148,149). In positive ion mode, retinyl ester (e.g., retinyl acetate) loses the fatty acid moiety and forms a dehydrated retinol fragment [M+H] at m/z 269.2 (148). However, carotenoids such as β-carotene showed only the protonated ion
[M+] at m/z 536.4 without fragmentation due to the absence of heteroatoms (e.g., oxygen for protonation) (149,150).

A potential limitation of using electrospray ionization for the analysis of vitamin A is its limited dynamic range (133,148). A dynamic linear range is the linear range where the response recorded is directly proportional to the analyte concentration (151). The dynamic range of electrospray ionization is greatly influenced by the ionization efficiency. Other than the electrospray operation conditions, factors that affect the ionization efficiency include the type of solvent (152), the buffer additive (153) and the occurrence of charge competition at the source due to the flow rate of the eluent (154). It has been suggested that the improved dynamic range of high-resolution mass analyzer (HRMS) can be impacted by electrospray ionization (155). Hence, one of the goals of our study was to improve the ionization efficiency of electrospray through the optimization of solvent compositions and buffer additives.

**Agilent jet stream electrospray ionization**

Multiple electrospray ion source designs (e.g., Standard ESI, Jet Stream ESI, viz Turbo Ion Spray, etc.) have been proposed to improve the performance of ionization by providing a higher selectivity and sensitivity (156). Among many, the global analytical instrumentation manufacturing company Agilent Technologies has introduced a new source design with improved sensitivity to meet the high throughput performance. Agilent jet stream (AJS) technology highlighted the improvement of desolvation (i.e., removal of solvent from material in solution) through two features: 1) application of sheath gas and 2) heated drying gas. Sheath gas is applied parallel to the nebulizer spray, which helps to concentrate the ion density for the capillary. Whereas the heated drying gas on the capillary further enhances the desolvation process to reduce possible background noise during ion formation (157). AJS ESI has claimed to enhance the instrument sensitivity 5- to10-fold as compared to conventional ESI (157). Given
the above-mentioned features, we decided to optimize the AJS source parameters and explore the factors that influence ESI ionization efficiency. Our goal is to improve the performance (i.e., dynamic range and instrument sensitivity) of HPLC-ESI(+-)HRMS-QTOF for the detection of α-retinyl esters, retinyl esters, α- and β-carotene in postprandial triacylglycerol-rich lipoprotein fractions.

**Factors affecting ionization efficiency**

Ionization efficiency is measured based on the ratio of the molecular ions formed to the molecules consumed in an ionization process (158). A desirable ionization efficiency for mass spectrometry is when an adequate amount of molecular ions are produced in the ion source for precise mass detection on the mass analyzer to produce high sensitivity and selectivity. The overall ionization process of electrospray is extremely complex and it is beyond the scope of discussion here (159). However, one common understanding of the requirement of electrospray ionization is the formation of ionized analytes in the liquid phase. Hence, the characteristics of the mobile phase and buffer additives for proper ionization are important.

The mobile phase composition and the buffer additive can affect the ionization efficiency in LC-MS analysis (153,160,161). The characteristics of mobile phases such as solubility, conductivity, ionic strength, viscosity, and the pH of buffer additives have the potential to influence the efficiency of a molecule forming charged droplets in the ESI and the signal response. In general, the selection of mobile phases and additives must allow chromatography separation and promote charge formation. In reversed-phase LC-based vitamin A analysis, methanol and methyl tert-butyl ether (MTBE) are two solvents commonly used for chromatography separation in reversed-phase C30 Carotenoid column. It has been previously concluded that these solvents for carotenoid analyses are suitable for LC-MS analysis using
atmospheric pressure chemical ionization (APCI) ion source (148). To our best knowledge, no reports have examined the mobile phase compatibility with electrospray ionization for vitamin A analysis.

LC-MS plays a critical role in the analysis of lipids (i.e., lipidomics) including complex lipid profiling. LC-MS-based lipidomic analyses shared a similar extraction protocol with vitamin A analysis, where it normally begins with the extraction of lipids (vitamin A) from the biological samples (triacylglycerol-rich lipoprotein fractions) before subjecting the samples to the column for chromatography separation and then to the mass spectrometer for identification and quantitation. The typical lipidomic analysis uses reversed-phase LC-MS with mobile phases systems that are compatible with ESI (+) (146). Common solvents for LC-MS-ESI (+) are volatile polar solvents desirable for stable electrospray formation.

Therefore, we decided to adopt the solvents commonly used in LC-MS based lipidomic method in the analysis of vitamin A in LC-ESI(+)–HRMS–QTOF (Table 4). An ideal electrospray solvent composition for stable spray formation requires at least 50% of conductive solutions that are polar organic solvent (e.g., methanol or acetonitrile) (145). In general, the relative polarity of a solvent is exhibited by the dielectric constant. Dielectric constant indicates the ability of a solvent to be polarized by the applied electric field in a vacuum and a value of less than 15 is considered nonpolar. Nonpolar solvents are also characterized as low conductivity. For instance, the strong polarity of water shows a relative polarity of 1.0 and a dielectric constant at 80, whereas nonpolar solvent MTBE has a relative polarity of 0.124 and a dielectric constant of 2.6. Hence, the nonpolar nature of MTBE can limit the ionization efficiency in electrospray due to its poor conductivity and low dielectric constant (162).
Because MTBE is not an ESI compatible solvent for stable spray formation, we propose to use isopropanol, the common solvent used in lipidomics, as a substitution to reduce the percentage of MTBE in the mobile phase composition. A small percentage of MTBE is required to achieve optimal chromatographic separation of carotenoid on the C30 Carotenoid column. The high viscosity and low volatility properties of isopropanol must be used in combination with other organic solvents such as acetonitrile (a dipole moment of 3.5 suitable for ion formation) for the generation of stable system backpressure and ion formation on the LC-MS. Hence, our new proposed mobile phase will contain a mixture of isopropanol, acetonitrile and MTBE.

**Table 4.** Selected properties of solvents used in LC-MS-based lipidomic and vitamin A analyses (163)

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Dipole moment (D)</th>
<th>Dielectric constant</th>
<th>Relative polarity</th>
<th>Viscosity 10-3 Pa s</th>
<th>Solubility in water g/100g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetonitrile</td>
<td>3.5</td>
<td>37.5</td>
<td>0.460</td>
<td>0.34</td>
<td>Miscible</td>
</tr>
<tr>
<td>Isopropanol</td>
<td>1.66</td>
<td>19</td>
<td>0.546</td>
<td>2.07</td>
<td>Miscible</td>
</tr>
<tr>
<td>Methanol</td>
<td>1.6</td>
<td>33</td>
<td>0.762</td>
<td>0.54</td>
<td>Miscible</td>
</tr>
<tr>
<td>Methyl-t-butyl-ether</td>
<td>1.4</td>
<td>2.6</td>
<td>0.124</td>
<td>0.36</td>
<td>4.8</td>
</tr>
<tr>
<td>Water</td>
<td>1.85</td>
<td>80.1</td>
<td>1.000</td>
<td>0.89</td>
<td>Miscible</td>
</tr>
</tbody>
</table>

In RPLC, buffer additives are added to maintain mobile phase pH for better compound retention during chromatography separation (164). The presence of ionizable compounds in the analysis is very sensitive to pH changes in the mobile phase and their retention times become unstable when pH is near the pKa; buffer additive with a pH closer to the pKa value of the acid/base allows the greatest buffering capacity to maintain the pH changes. A pKa value is a quantitative information to measure the strength of an acid/base. In MS analysis with any source
of ionization technique, buffer additives are added to promote and stabilize ionization. In general, ionization generates primarily molecular ions \([M^+]\), protonated molecules \([M + H]^+\), adduct ions, and ions with simple losses such as molecule losing a water molecule \([M + H – H_2O]^+\). These molecules are formed in the presence of various proton donors in a pH-specific environment, e.g., of proton donors are ammonium ion from a salt-based buffer or hydronium ion in an acidic buffer. Therefore, a buffer is added to MS analysis to stabilize the ionizable compounds and to maximize the sensitivity by forming the appropriate ions for MS detection. The examples of the buffer used in LC-MS analysis can be salt-based (e.g., ammonium formate) or acid-based (formic acid). These buffers are added are usually added at the concentration range from 5 to 10 mM for salt-based and 0.05 – 0.2% for acid-based (146).

Cajka et al reported a systematic evaluation of various mobile phase buffers in RPLC-MS-based lipidomic and found that buffers are an important modifier to improve LC separation, ionization and lipid detection (165). Cajka and others observed that ammonium formate provides an optimized intensity of neutral lipids (165). In contrast, it has been reported that ammonium compounds can suppress the ionization of phosphatidylcholine, a neutral lipid, in ESI-MS (166). These studies suggested that the role of buffer additive could be mobile phase and molecule specific. In an LC-ESI(+) MS/MS-based fat-soluble vitamin analysis, 0.1 % formic acid and 5 mM ammonium formate were used as a modifier to increase instrument sensitivity (167). These studies have contributed to the basis of our decision to explore the buffer additives options (e.g., 0.1 % formic acid, 0.1% formic acid and 5 mM ammonium formate, and 5 mM ammonium formate) to improve the detection of \(\alpha\)-retinyl esters and retinyl esters in the LC-ESI(+) HRMS-QTOF. We speculate that by modifying the buffer additive, we can optimize the charge competition between analyte within the sample matrix for optimal signal enhancement of the
target molecules. However, the mode of action of the relationship between ionization and buffer additive is beyond the scope of our study. Buffer additive containing salts is not soluble in the nonpolar organic solvents such as methyl-\textit{ tert}-butyl ether (MTBE) and isopropanol, thus may cause precipitation. Therefore, the gradient transition between the salt-containing mobile phase to the salt-free mobile phase must be carefully designed to avoid salt precipitation in the HPLC system, which may cause system overpressure.

Table 5. Volatile buffers compatible with LC-MS (164)

<table>
<thead>
<tr>
<th>Volatile buffer</th>
<th>Formula</th>
<th>$pK_a$</th>
<th>Buffer range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium formate</td>
<td>HCO$_2$NH$_4$</td>
<td>3.8</td>
<td>2.8-4.8</td>
</tr>
<tr>
<td>Formic acid</td>
<td>HCO$_2$H</td>
<td>3.8</td>
<td>-</td>
</tr>
</tbody>
</table>

In chapter 3, we will focus on the optimization of the HPLC-ESI(+) -HRMS-QTOF method with enhanced sensitivity for the detection of provitamin A carotenoids and the retinyl ester bioconversion products of $\alpha$- and $\beta$-carotene. We attempt to develop a novel solvent composition that is ESI-compatible. We hypothesize that the sensitivity of the existing LC-MS method published in the literature for provitamin A analysis can be optimized by changing the compositions of mobile phases and buffer additives compatible with the ion source. We will describe the use of HPLC-HRMS-QTOF with Agilent Jet Stream ESI technology and the optimization of source parameters (i.e., sheath gas temperature and flow) specific to Agilent Jet Stream ESI technology. We intend to use the novel HPLC-HRMS-QTOF method we developed to evaluate the bioefficacy of the provitamin A carotenoid biofortified banana fruit. The HPLC-ESI(+) -HRMS-QTOF method will be used to directly measure the postprandial appearance of the bioconversion products (i.e., $\alpha$-retinyl esters and retinyl esters) formed after the ingestion of provitamin A carotenoid biofortified bananas (Chapter 4).
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CHAPTER 3. HIGH-RESOLUTION MASS SPECTROMETRIC ANALYSIS FOR THE QUANTITATION OF THE POSTPRANDIAL PLASMA APPEARANCE OF PROVITAMIN A CAROTENOIDS AND THEIR RETINYL ESTER BIOCONVERSION PRODUCTS

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Abstract

The asymmetrical provitamin A carotenoid α-carotene is enzymatically cleaved to retinol and α-retinol. Previous reports highlighted the necessity for excluding the biologically inactive α-retinol when quantifying in plasma the retinol bioconversion products of ingested α-carotene. High-resolution mass spectrometry (HRMS) has continuously evolved to become a sensitive and reliable tool for both qualitative and quantitative analysis. We developed a highly selective and sensitive HPLC-ESI(+)–QTOF-HRMS method to simultaneously quantify provitamin A carotenoids and their retinyl ester and α-retinyl ester bioconversion products in postprandial plasma. To develop the method, we used plasma triacylglycerol-rich lipoprotein (TRL) fractions isolated from postprandial plasma after research participants consumed a test meal of cooked carrots. The intact α- and β-carotene, α-retinyl ester bioconversion products of α-carotene, and retinyl ester bioconversion products of both α- and β-carotene were extracted from the TRL fractions. Labeled internal standards (d8-α-retinyl palmitate, 13C10 β-carotene, 13C10 retinyl
palmitate) were added to correct for plasma matrix effects. The TRL extracts were analyzed by using HPLC-ESI(+)-QTOF-HRMS and the exact mass extracted ion chromatograms (EICs) were generated in full-scan acquisition mode. We developed an ESI(+-) compatible mobile phase gradient that stabilized the operational pressure of the HPLC-ESI(+)-QTOF-HRMS system and enhanced sensitivity. The following mobile phase modifiers were compared: 0.1% formic acid, 0.1% formic acid plus 5 mM ammonium formate, and 5 mM ammonium formate. A column wash system was used between sample injections to minimize on-column matrix accumulation. The optimized HPLC-ESI(+)-QTOF-HRMS method decreased by up to 100-fold the limits of detection (LOD) and limits of quantitation (LOQ) for α-retinyl palmitate (α-RP), retinyl palmitate (RP), α-carotene and β-carotene as compared with more conventional HPLC-tandem mass spectrometric (LC-MS/MS) methods. The method also extended the linear calibration range of the analytes, including α-carotene, β-carotene, α-retinyl palmitate, retinyl palmitate. When evaluating precision, the intra- and inter-day relative standard deviation (RSD%) values for all calibration standards were less than 10%. Within the plasma TRL matrix, the intra-day precision (RSD%) values for α-carotene, β-carotene, and retinyl palmitate were 10%, 8%, and 5%, respectively. In conclusion, we developed an ultra-high-sensitivity HPLC-ESI(+)-QTOF-HRMS method for the quantitation of fmol amounts of provitamin A carotenoids and their vitamin A bioconversion products in human plasma TRL fractions. The method is suitable for measuring the bioavailability and bioconversion to vitamin A of the provitamin A carotenoids consumed in plant matrices, such as carrots or provitamin A-biofortified foods.

**KEYWORDS:** α-carotene, α-retinol, bioconversion, bioavailability, electrospray ionization, high-resolution, mass spectrometry, quadrupole-time-of-flight, retinyl palmitate

**ABBREVIATIONS:** BHT, butylated hydroxytoluene; CV, coefficient of variation; ESI,
electrospray ionization; HRMS, high-resolution mass spectrometry; MTBE, methyl-tert-butyl ether; QTOF, quadrupole-time-of-flight

**Introduction**

Vitamin A deficiency remains prevalent in regions where plant-based sources account for the majority of dietary vitamin A\(^1\)–\(^3\). Both \(\alpha\)- and \(\beta\)-carotene are provitamin A carotenoids richly found in fruits and vegetables and metabolized to produce vitamin A. During central cleavage, whereas \(\beta\)-carotene (with two \(\beta\)-ionone rings) produces two retinols, \(\alpha\)-carotene (with one \(\beta\)-ionone and one \(\varepsilon\)-ring) yields only one retinol and one biologically inactive \(\alpha\)-retinol\(^4\).

Analogous to retinol, \(\alpha\)-retinol is esterified with a fatty acid moiety within the intestinal mucosa and circulated in the plasma triacylglycerol-rich lipoprotein (TRL) fractions as an \(\alpha\)-retinyl ester. In regard to the challenge of resolving the isomeric \(\alpha\)-retinyl esters and retinyl esters with reversed-phase chromatography\(^5\)–\(^7\), Goetz et al.\(^8\) recently published a liquid chromatography-tandem mass spectrometric (LC-MS/MS) analytical method that allows the identification and exclusion of \(\alpha\)-retinyl esters when quantifying retinyl esters in the plasma of participants who consumed \(\alpha\)-carotene-containing foods. Subsequently, Cooperstone et al.\(^9\) reported large inter-individual variability in the conversion efficiency of dietary \(\alpha\)-carotene to vitamin A. These studies highlighted the need for highly sensitive LC-MS instrumentation for direct measurement of the biological appearance of \(\alpha\)-carotene and its \(\alpha\)-retinol derivatives, i.e., \(\alpha\)-retinyl esters, to avoid overestimation of the vitamin A contribution from \(\alpha\)-carotene in \(\alpha\)-carotene-containing foods.

The recent evolution of high-resolution mass spectrometry (HRMS) technology (e.g., quadrupole-time-of-flight or Orbitrap) with enhanced mass resolution has advanced the capacity for qualitative and quantitative analyses\(^10\)–\(^12\). Mass resolution, as measured by resolving power,
refers to the ability to separate two mass spectral peaks of equal height and width. HRMS is capable of distinguishing isobaric compounds with similar nominal masses; high mass resolution MS allows exact mass measurement of a compound with a narrow mass tolerance error. When coupled with liquid chromatography, HRMS is a selective and sensitive qualitative and quantitative tool for postprandial plasma metabolites\textsuperscript{12,13}. In contrast to conventional LC-MS/MS analysis based on selected reaction monitoring (SRM) acquisition mode, LC-HRMS utilizes full-scan acquisition mode to record all ions within the sample as a total ion current (TIC) chromatogram to enable comprehensive sample description. LC-HRMS offers retrospective investigation for both targeted and untargeted analyses without re-injection of sample. Due to its versatility to measure hundreds of endogenous metabolites within a sample, there is a paradigm shift from using LC-MS/MS-based SRM to LC-HRMS full-scan acquisition mode\textsuperscript{10,11}. Moreover, the previously limited linear dynamic range has been extended in modern HRMS instrumentation\textsuperscript{14}. In fact, current HRMS systems are reported to have equivalent or better performance than offered by conventional MS/MS quadrupole mass analyzers\textsuperscript{11–13,15,16}.

Electrospray ionization (ESI) is the most widely used soft-ionization technique in LC-MS-based metabolomic studies\textsuperscript{17}. LC-ESI-MS has been previously applied in quantifying carotenoids and vitamin A in human serum but with a limited dynamic range as compared with atmospheric pressure chemical ionization (APCI)\textsuperscript{18,19}. With the growing understanding of the ESI ionization mechanism, the extension of linear dynamic range can be achieved through the improvement of ionization efficiency and the efficiency of transferring gas-phase ions into the mass analyzer\textsuperscript{20}. During electrospray, the formation of charged droplets and the desolvation processes affect the formation of molecular ions. These mechanisms are greatly affected by the flow rates, solvent properties, source parameters, and source type\textsuperscript{21}. Specifically, the choice of
solvent can greatly affect the dynamic range and its sensitivity. Because the commonly-used mobile phase for carotenoid and vitamin A reversed-phase LC analysis is not compatible with ESI-MS, choosing the appropriate mobile phase and buffers to support ionization for LC–ESI–HRMS analysis is important to achieve high sensitivity.

In an HPLC-ESI-MS-based analytical method, matrix effects are a common source of systematic error in quantitative analysis. Matrix effects are the suppression or enhancement of analyte signal response due to the presence of coeluting substances within a biological matrix (e.g., plasma). As lipid-soluble vitamins in human plasma are generally extracted through liquid-liquid extraction (LLE) with organic solvents, the coextraction of a wide spectrum of endogenous plasma lipids is unavoidable. Phospholipids are known to cause matrix effects that affect the reproducibility, linearity, and accuracy for consistent quantitation in LC-MS analysis. The unavoidable matrix effects have highlighted the importance of isotope-labeled internal standards to correct for the signal variability. Another strategy to minimize the coeluting effects is the use of a post-separation by-pass wash method. After compound elution from the chromatographic column, a column wash system is implemented and the LC wash eluant is diverted to waste, which reduces both the accumulation of matrix lipids on the analytical column and the contamination of lipids in the mass spectrometer.

The aim of this study was to develop and validate an LC-ESI-HRMS method to simultaneously quantify α-carotene, β-carotene, α-retinyl ester, and retinyl ester bioconversion products present in postprandial plasma after consuming provitamin A-containing foods. We emphasized the use of a column washing by-pass method and implemented the corresponding stable isotope-labeled internal standards to compensate for matrix effects. To the best of our knowledge, this is the first report of simultaneous quantitation of plasma provitamin A
carotenoids and their bioconversion products using LC-ESI-HRMS technology. The ultra high-sensitivity method is suitable for measuring the postprandial plasma appearance of provitamin A carotenoids consumed in plant matrices, such as carrots or biofortified foods, as well as the vitamin A bioconversion products.

Materials and methods

Chemicals and reagents. The following chemicals were purchased from Fisher-Scientific (Pittsburgh, PA, USA): acetonitrile (Optima, LC/MS), methanol (Optima, LC/MS), 2-propanol (Optima, LC/MS), hexane (Optima, LC/MS), water (Optima, LC/MS), ammonium formate (HPLC grade), and formic acid aqueous solution (HPLC grade). Methyl tert-butyl ether (HPLC Plus) and 200 proof ethyl alcohol (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO). α-Carotene was purchased from CaroteNature (Lupsingen, Switzerland) for use as a calibration standard; β-carotene and retinyl palmitate (RP) were purchased from Sigma-Aldrich; retinyl linoleate (RLO), retinyl oleate (RO) and 9-cis retinyl stearate (9-cis-RS) were purchased from Toronto Research Chemicals (North York, Ontario, Canada). Due to chemical impurity of the all-trans-retinyl stearate calibration standard, 9-cis-RS was used as an alternative. The following standards were purchased from Cambridge Isotopes (Andover, MA): α-retinyl palmitate, d8-α-retinyl palmitate (10,14,19,19,20,20,20-[2H8]-α-retinyl palmitate) (96%), and 13C10-β-carotene (12,12′,13,13′,14,14′,15,15′,20,20′-[13C10]-all trans-β-carotene) (99%), and 13C10-retinyl palmitate (8,9,10,11,12,13,14,15,19,20-[13C10]-retinyl palmitate).

Preparation of standard and stock solutions. Calibration standards were used to construct calibration curves. Stock and working solutions of α-retinyl palmitate, retinyl palmitate, retinyl linoleate, retinyl oleate, 9-cis retinyl stearate, α- and β-carotene were prepared in hexane containing 1 g butylated hydroxytoluene (BHT)/L. Stock and working solutions of the
internal standards \( \delta_8\)-\( \alpha \)-retinyl palmitate, \(^{13}\)C\(_{10}\)\(-\beta\)-carotene, \(^{13}\)C\(_{10}\)-retinyl palmitate were prepared in hexane and 200 proof ethyl alcohol containing 1 g BHT/L, respectively. The absolute concentration of each standard in the working solution was confirmed by spectrophotometer using the published absorption coefficients (\(E_{1\%}\))\(^2\). Because the wavelength of maximum absorbance (\(\lambda_{\text{max}}\)) for the retinyl esters is not affected by their fatty acid moieties, and retinyl esters and retinol have identical molar absorptivity in a given solvent, the \(E_{1\%}\) of retinyl palmitate was used for all retinyl esters\(^3\). The ratio of the published \(E_{1\%}\) of all\(-\)trans-retinol to that of all\(-\)trans-retinyl palmitate dissolved in a similar solvent was used as a basis to estimate the \(E_{1\%}\) of \(\alpha\)-retinyl palmitate. Based on the relationship between molar extinction coefficient and percent extinction coefficient\(^4\), we calculated the \(E_{1\%}\) of \(\alpha\)-retinyl palmitate in ethanol as 842 measured at \(\lambda_{\text{max}}\) of 311 nm.

**Collection of postprandial plasma.** Postprandial blood samples were collected from healthy research participants recruited according to a protocol approved by the Iowa State University Institutional Review Board (IRB # 18-250-00). The participants consumed a cooked carrot test meal (350-400 g) with added peanut oil (12 g). Blood samples were collected at selected time points and plasma was separated. Plasma triacylglycerol-rich lipoprotein (TRL) fractions were isolated by cumulative rate ultracentrifugation, as previously described\(^5\), and stored at \(-80^\circ\)C until analyzed.

**Extraction of carotenoids and retinyl esters from the plasma triacylglycerol-rich lipoprotein fractions.** The carotenoids and retinyl esters were extracted from the TRL fractions by deproteination with an equal volume of 200 proof ethyl alcohol (1 g BHT/L). The fractions were then spiked with the appropriate amounts of the following internal standards in 200 proof ethyl alcohol: \( \delta_8\)-\( \alpha \)-retinyl palmitate, \(^{13}\)C\(_{10}\)-retinyl palmitate, and \(^{13}\)C\(_{10}\)-\( \beta \)-carotene. The
Carotenoids and retinyl esters were then extracted three times with hexane containing 1 g BHT/L followed by centrifugation. The combined supernatants were transferred to a glass test tube and dried under vacuum. The dried extracts were reconstituted with 60 µL MTBE:methanol (1:1, v/v). All extraction procedures were carried out under dim yellow light.

**HPLC-ESI(+)QTOF-HRMS.** The HPLC-ESI(+)-QTOF-HRMS system consisted of an Agilent 1290 Infinity LC system interfaced with an Agilent 1260 Diode Array Detector (DAD) and an Agilent 6540 Ultra High-Definition (UHD) Accurate-Mass Quadrupole-Time-of-Flight (QTOF) mass spectrometer equipped with dual Agilent Jet Stream Thermo Gradient Focusing Technology electrospray ionization source (dual AJS ESI) (Agilent Technologies). The analytical column was a C30 Carotenoid Column (4.6 mm × 250 mm, 3 µm) (YMC, Inc.) coupled to a guard column (4.0 mm × 23 mm, 3 µm) (YMC, Inc.) and protected by an inline filter. The autosampler and the analytical column were maintained at 4 °C and 40 °C, respectively. The dual AJS ESI was operated in positive ion mode. The source conditions were as follows: gas temperature 350 °C, drying gas 12 L/min, nebulizer gas 45 psi, Vcap 3500 V, nozzle voltage 250 V, sheath gas temperature 400 °C, and sheath gas flow 12 L/min. For the MS TOF settings, the fragmentor was set at 150 V, skimmer at 50 V, and OCT 1 RFVpp at 625 V. Total ion current (TIC) chromatograms were acquired using HRMS full-scan acquisition mode over a mass range of 110-1700 m/z at a rate of 1 spectrum/s. The Q-TOF-MS system was calibrated and tuned using ESI tuning mix (Agilent Technologies) at 4 GHz extended dynamic range mode over a range of 0-1700 m/z. The instrument mass resolution was maintained at or greater than 30,000. An internal lock mass mixture was prepared in 95% acetonitrile and was infused for constant mass correction: m/z 322, m/z 622, and m/z 1221.
**Compound Identification.** HRMS was used to tentatively identify all provitamin A and vitamin A-related compounds present in plasma within an optimal mass extraction window (MEW) established during method development. The extracted ion chromatogram (EIC) of the targeted analyte was generated post-data acquisition from the TIC chromatogram. Post-acquisition data processing of the targeted metabolites was completed with the Agilent MassHunter Workstation Quantitative analysis for Q-TOF (version 10.0). All chromatograms were obtained from the Agilent MassHunter Workstation Qualitative Analysis for Q-TOF (version B.10.0).

**Optimization of mobile phase composition for HPLC-ESI(+)-QTOF-HRMS performance.** An isopropanol-based ESI(+) compatible mobile phase system (isopropanol/acetonitrile/MTBE) was developed and compared with a conventional MTBE-based non-ESI Compatible mobile phase system (MTBE/methanol/water) for reversed-phase carotenoid and vitamin A analysis. The gradient and flow rates for each mobile phase system were optimized to provide optimal separations of α-carotene, β-carotene, α-retinyl esters, and retinyl esters in plasma TRL fractions. Both mobile phase systems contained 0.1% formic acid (v/v). In the binary pump system, channels A1 and B1 of the MTBE-based non-ESI compatible mobile phase system contained solvent A1: 90:10 methanol/water and solvent B1: 78:20:2 MTBE/methanol/water. The following gradient was used: 0-5 min, 40% solvent B1 at 1.1 mL/min, 5-17 min, 40% to 47% solvent B1 at 1.2 ml/min, 17-27 min, 47% to 52% solvent B at 1.2 ml/min, 27-35 min, 52% to 54% solvent B at 1.2 ml/min, 35-39 min, 54% to 100% solvent B at 1.2 ml/min, holding at 100% solvent B1 for 6 min at 1.2 ml/min, and re-equilibrating at 40% solvent B1 for 5 min at 1.1 mL/min. The flow rate was adjusted to avoid excessive back pressures due to the effects of the methanol. In channels A2 and B2 of the binary pump, the
isopropanol-based ESI-compatible mobile phase system contained solvent A2: 65:25:10 methanol/acetonitrile/water (v/v) and solvent B2: 50:25:25 isopropanol/acetonitrile/MTBE. The following gradient was used: 0-22.5 min, 25% to 70% solvent B2, 22.5-36.5 min, 70% to 90% solvent B2, 36.5-40 min, 90% to 100% solvent B2, holding at 100% solvent B2 for 4 min, and re-equilibrating to 25% solvent B2 for 5 min. The flow rate was constant at 1.2 mL/min.

**Optimization of buffer additives for HPLC-ESI(+)-QTOF-HRMS performance.**

The addition of buffers to the isopropanol-based ESI-compatible mobile phase system was investigated to optimize the sensitivity for the detection of the provitamin A carotenoids and retinyl esters. The following buffer additives were compared: 0.1% formic acid, 0.1% formic acid plus 5 mM ammonium formate, and 5 mM ammonium formate. To evaluate the effect of the buffers on the detection sensitivity for the provitamin A carotenoids and retinyl esters, plasma TRL fractions were spiked with known amounts of α-retinyl palmitate, retinyl palmitate, and β-carotene. The peak area for each spiked analyte was compared across the buffer additives.

**Optimization of chromatographic precision.** A post-separation by-pass wash method was implemented after each sample elution from the chromatographic column to reduce the accumulation of the plasma matrix on the analytical column and then divert the LC wash flow to waste. This approach reduced the on-column accumulation of strongly retained plasma matrix components between injections. In addition, a multistep gradient elution program was introduced to better resolve the late-eluting α- and β-carotene from co-eluting phospholipids and other matrix components. The precision expressed as the relative standard deviation (RSD%) of the analyte concentrations in the quality control plasma TRL fraction was used to determine the effectiveness of the chromatographic elution conditions.
**Method validation.** The quality parameters established for validation of the HPLC-ESI(+)–QTOF-HRMS method were selectivity, linear dynamic range, limit of detection (LOD), limit of quantitation (LOQ), and precision. Selectivity was achieved using a combination of the retention time from the chromatographic separation and the HRMS exact mass measurement of the precursor ions on the EICs using an optimal narrow mass extraction window (MEW). To evaluate the linear dynamic range, calibration curves were prepared by serial dilution to produce at least eight concentrations of each authentic standard, including α- and β-carotene, α-retinyl palmitate, and retinyl palmitate. The on-column amounts injected ranged from 0.0635 to 2,005 pmol of α-retinyl palmitate, 0.03465 to 2,100 pmol of retinyl palmitate, 0.043 to 3,896 pmol of α-carotene, and 0.338 to 3,812 pmol of β-carotene. Each calibration curve was constructed by linear regression using the response ratio of analyte and internal standard (A<sub>a</sub>/A<sub>IS</sub>) plotted against the injected amount of the analyte. Each acceptable calibration curve attained a correlation coefficient (r<sup>2</sup>) of 0.990 or greater. The LOD and LOQ values of the target analytes were the lowest concentrations injected that give a signal-to-noise (S/N) ratio of 3 and a S/N ratio of 10, respectively.<sup>33</sup>

The precision of the method was evaluated by using replicate analyses of the following: 1) pure calibration standards, 2) a quality control plasma TRL pool isolated after research participants consumed a carrot test meal, and 3) postprandial response curves using plasma TRL fractions isolated at multiple time points after participants consumed a carrot test meal. For the intra-day precision of the standard solution, relative standard deviations (RSD%) for increasing concentrations of α-retinyl, retinyl palmitate, α- and β-carotene were reported. This experiment was repeated on two additional days to evaluate inter-day precision. For the intra-day precision of the plasma TRL, replicates of pooled QC extracts (n = 7) were analyzed on the same day, and
RSD% for α-retinyl palmitate, retinyl palmitate, α- and β-carotene were calculated. For the evaluation of the inter-day precision of the plasma TRL analyses, three sets of postprandial response curves after participants consumed a carrot est meal were used. Plasma samples at each time point were pooled across participants prior to isolation of the TRL fraction.

Results and discussion

Optimization of HPLC-ESI(+)-QTOF-HRMS conditions. The purpose of this study was to establish a highly sensitive HPLC-ESI(+)-QTOF-HRMS method suitable for simultaneous quantitation of provitamin A carotenoids and their retinyl ester bioconversion products in postprandial plasma. A mobile phase consisting of methanol, water, isopropanol, acetonitrile, and lesser amounts of MTBE was selected for compatibility with electrospray mass spectrometry. The goal of this novel mobile phase system was to improve the overall LC-MS instrumentation performance, including the electrospray ionization efficiency. We observed an overall LC-MS system improvement regarding the system operating pressure. By using our isopropanol-based ESI(+)-compatible mobile phase system, we achieved near-constant backpressure (Figure 1) at a flow rate of 1.2 ml/min at 40 °C during the separation of provitamin A carotenoids and retinyl esters in plasma TRL on a C30 Carotenoid Column. Operating the system within its pressure limit under constant pressure protects the analytical column and increases the stability of the LC-MS instrumentation for maximum efficiency.

Chemical background noise ions are by-products of soft ionization techniques such as ESI, which is highly efficient in producing intact ions of contaminants found in the mobile phases\textsuperscript{34}. In mass spectrometry, these ionizable contaminants and solvent clustering contribute to the increase in chemical background noise during analysis\textsuperscript{35}. As the total ion current (TIC) chromatogram provides the summed intensity of all ionized compounds and contaminants across
the entire mass range detected, a high chemical background noise contributes to the high and drifting TIC baseline, which affects the S/N ratio. Herein, we used a novel mobile phase composition to reduce the chemical noise by producing a stable spray in the ESI positive ion mode. We observed a lower chemical background noise in the TIC chromatogram of the isopropanol-based ESI(+) compatible mobile phase system as compared with the MTBE-based non-ESI(+) compatible mobile phase system (Figure 2). During the preliminary assessment, the sensitivity of the novel mobile phase combination on ionization efficiency was studied by comparing the S/N ratio improvement for plasma TRLs spiked with retinyl palmitate, retinyl stearate, α- and β-carotene (Figures 3 and 4). In summary, background noise reduction was achieved using the isopropanol-based ESI(+) compatible mobile phase system, which increased the sensitivity in quantitation of carotenoids and vitamin A molecules, including within the plasma TRL matrix.

The isopropanol-ESI-compatible mobile phase system was therefore used as the final mobile phase system to evaluate the effect of buffer additives on signal intensity. Buffer is added to the mobile phase system to increase conductivity and facilitate protonation. We evaluated the influence of different buffer additives on the signal intensities of spiked α-retinyl palmitate, retinyl palmitate, and β-carotene in plasma TRLs. While parameters such as LC gradient, flow rate, and column temperature were kept constant, we compared the relative peak area responses of the spiked α-retinyl palmitate and retinyl palmitate in the TRL extracts isolated from a plasma pool. The peak areas of the spiked α-retinyl and retinyl palmitate in the plasma TRLs were significantly higher in both ammonium formate-containing mobile phases ($P < 0.05$) (Figure 5). For simplicity, the 5 mM ammonium formate buffer was chosen as the buffer
additive for the isopropanol-based ESI-compatible mobile phase system adopted for the analysis of provitamin A carotenoids and retinyl esters in the plasma TRL fraction.

While sample clean-up methods using liquid-liquid extraction and solid-phase extraction are often used to minimize matrix effects contributed by the presence of plasma phospholipids, the recovery of analytes would not be expected to meet the needs of our ultra high-sensitivity analyses. Thus we utilized a chromatographic approach to reduce matrix effects. We optimized the elution conditions using: 1) a post-separation by-pass wash method to clean the column and divert the eluted matrix to waste after each sample elution, and 2) a multistep gradient elution program to increase the separation of α- and β-carotene from the interfering compounds in the plasma TRL matrices. These efforts minimize ion source contamination and remove the residual matrix from the chromatographic column. The effectiveness of the chromatographic elution conditions was assessed through the precision of replicate analyses of the quality control plasma TRL pool (Table 1). Our preliminary assessment indicated that the by-pass wash method improved the RSD% for α-retinyl and retinyl palmitate, but not for late-eluting α- and β-carotene. To increase the separation of α- and β-carotene from the interfering compounds, we implemented a multistep separation gradient program. Combining these two approaches yielded RSD% < 10 % for α- and β-carotene in a precision test using pooled QC samples. The RSD% in plasma TRLs reported in the present study are within the acceptable specification. The improved precision likely resulted from the separation of background interfering compounds from the target analytes by the multistep gradient, which was particularly important for α-carotene and β-carotene based on their late elution times.

**Internal standard selection.** The occurrence of matrix effects in plasma affects quantitative analysis in LC-MS. The degree of ion suppression/enhancement for a given analyte
is sample- and instrument-dependent. In addition, fat-soluble vitamins and associated lipids present in human plasma often coelute in reversed phase chromatography. Therefore, it is advisable to use labeled internal standards to compensate for signal variation in the presence of biological matrices. To mimic the behavior of the target analytes, we used stable isotope-labeled internal standards. We used $d_8$-$\alpha$-retinyl palmitate as the internal standard for all $\alpha$-retinyl esters, $^{13}$C$_{10}$-retinyl palmitate as the internal standard for all retinyl esters, and $^{13}$C$_{10}$-$\beta$-carotene as the internal standard for $\alpha$- and $\beta$-carotene. The effectiveness of the internal standards was evaluated based upon the improvement of the RSD$\%$ in the precision assay. It was reported previously that biological matrix suppression was seen for native $\beta$-carotene and $^{13}$C$_{10}$-$\beta$-carotene but not for retinyl ester MS responses. We similarly observed that coeluting phospholipids were more problematic for the precision of quantitative analyses of the non-polar $\alpha$- and $\beta$-carotene. We successfully used stable isotope internal standards, in addition to optimal chromatographic separation and the post-separation by-pass column wash method, to compensate for the matrix suppression, as indicated by the improved RSD$\%$ in the precision test (Table 1).

**Method validation.** The selectivity, limit of detection, limit of quantification, linearity, and precision of the method were evaluated with the final mobile phase system.

**Selectivity.** To ascertain the selectivity of the analysis of the carotenoids and retinyl esters in plasma TRLs, we utilized chromatographic separation of the targeted molecules on a C$_{30}$ Carotenoid Column, and EICs extracted from the TIC chromatogram on HPLC-ESI(+) - QTOF-HRMS using an optimal mass extraction window (MEW). We identified the target analytes based on coelution with authentic standards. The HRMS full scan acquisition mode allowed the extraction of the targeted molecules with a MEW of $<10$ ppm around the
monoisotopic mass. We utilized the accurate mass identification provided by the high-resolving power of the QTOF. Although HRMS offers a narrow mass window extraction for low sub-ppm levels of mass deviation, choosing the optimal MEW value (in ppm) is compound-specific, and depends on the MS signal sensitivity, and the occurrence of matrix interferences. A good balance is required between selecting the optimal mass extraction window for selectivity without losing the MS signal sensitivity\textsuperscript{10,39}.

**Linear dynamic range and calibration curves.** The dynamic range was tested by assessing the linear relationship of the analyte concentration and the ion response (i.e., \(m/z\) 269 for retinoids and \(m/z\) 536+537 for carotenoids) on HPLC-ESI(+)QTOF-HRMS. The linear dynamic ranges for \(\alpha\)-retinyl palmitate, retinyl palmitate, \(\alpha\)- and \(\beta\)-carotene were evaluated by analyzing at least seven injected amounts of calibration standard. When the maximum amount injected on column was 2,000 pmol for \(\alpha\)-retinyl palmitate, retinyl palmitate, \(\alpha\)- and \(\beta\)-carotene, the HPLC-ESI(+)QTOF-HRMS dynamic range became nonlinear. This observation agrees with the previous studies that ESI has a limited linear dynamic range\textsuperscript{40,41}. We were able to extend the linear dynamic range by using the respective stable-isotope-labeled internal standards for \(\alpha\)-retinyl palmitate, retinyl palmitate, \(\alpha\)-carotene, and \(\beta\)-carotene. (The \(^{13}\)C-\(\beta\)-carotene internal standard was used as the internal standard for both \(\alpha\)- and \(\beta\)-carotene.) Calibration curves were constructed by plotting the response ratio of the analyte and the internal standard against the injected amount of analyte (Figure 6). Over the range evaluated (up to 2,000 pmol on column) for retinyl palmitate, \(\alpha\)- and \(\beta\)-carotene, positive ion ESI produced an excellent linear response with at least three orders of magnitude and a correlation coefficient \((r^2) \geq 0.99\). For the more minor plasma metabolite, \(\alpha\)-retinyl palmitate, the linear range extended to 780 pmol on column, which was the highest amount evaluated. Compared with a previous analysis of the linear
dynamic range for retinyl palmitate using quadrupole LC-MS coupled with electrospray ionization through flow injection, our data showed an improved linear dynamic range for retinyl palmitate. When $^{13}$C$_{10}$ retinyl palmitate was used as the internal standard for retinyl palmitate, the linear dynamic range was extended to 2,100 pmol on-column with an $r^2$ value of 0.9996. When $^{13}$C$_{10}$-β-carotene was used to correct the β-carotene response, the linear dynamic range was extended to 2,079 pmol on-column with an $r^2$ value of 0.9926. Previously, APCI-LC-MS produced a linear response of 0.25-1987 pmol/μL for β-carotene. Our linear dynamic ranges for retinyl palmitate and β-carotene were thus comparable with previous reports. We showed that the use of stable isotope-labeled internal standards extended the linear dynamic range for HPLC-ESI(+)-QTOF-HRMS. A limited linear dynamic range was previously identified as a limitation of ESI for the specific analytes targeted in this study. Our results highlighted the importance of using stable-isotope-labeled internal standards in correcting for the effects of the plasma matrix in suppressing the retinoid and carotenoid signal response.

**Limit of Detection (LOD) and Limit of Quantitation (LOQ).** The LODs and LOQs of HPLC-ESI(+)-QTOF-HRMS shown are presented as pmol injected onto the HPLC column. Our LOD and LOQ values of α-retinyl, retinyl palmitate, α- and β-carotene on the HPLC-ESI(+)-QTOF-HRMS were compared with those reported by others using HPLC-MS/MS and HPLC-MS. For α-retinyl palmitate, the LOD and LOQ values obtained in our study were 15-fold lower compared with the LC-MS/MS analyses of selected ions (SIM) reported by Goetz et al. The LOD and LOQ values of retinyl palmitate on HPLC-ESI(+)-QTOF-HRMS were reduced to an even greater extent; they were 100-fold lower than values reported by Goetz et al. using LC-MS/MS and van Breemen et al. using LC-MS. In comparison to the LC-MS/MS analyses by others, we obtained lower LOD and LOQ values for α-carotene and β-carotene by using
HPLC-ESI(+)-QTOF-HRMS. Our reported LOD and LOQ values for α- and β-carotene are comparable to those reported by Meulebroek et al. 44 using high-resolution Orbitrap analysis of α- and β-carotene. The authors also observed that high-resolution Orbitrap could achieve a higher sensitivity as compared to high resolution-Orbitrap-MS/MS analysis for α- and β-carotene. These findings support a greater or equivalent selective and sensitive full-scan acquisition capability offered by HRMS versus the conventional SRM scan mode on MS/MS by triple quadrupole mass analyzers.

**Precision.** The intra- and inter-day precision (RSD%) obtained with standard solutions are shown in Table 3. The intra-day precisions were 4.3%, 1.0%, 7.1%, and 1.4% for α-retinyl palmitate, retinyl palmitate, α-carotene, and β-carotene, respectively. The corresponding inter-day precisions were 7.7%, 5.2%, 0.9%, and 0.3%. The inter-day precisions of the postprandial TRL area under the absorption curves calculated by the trapezoidal method were 12.8%, 10.1%, 9.8%, and 8.0% for α-retinyl palmitate, retinyl palmitate, α-carotene, and β-carotene, respectively (Table 4).

**Appearance of carotenoids and their bioconversion products in postprandial plasma TRL.** Our developed and validated method was successfully applied to simultaneously measure the provitamin A carotenoids, α- and β-carotene, and their retinyl ester bioconversion products in postprandial plasma TRL fractions isolated after research participants consumed a cooked carrot test meal (Figure 7). During positive ion electrospray, both the α-retinyl esters and retinyl esters produced an abundant in-source dehydrated retinol fragment ion base peak at m/z 269.2 [MH - H2O]+, which was chosen for quantitation19. For the carotenoids, the positive ion electrospray produced unfragmented dominant molecular ion [M]⁺ of m/z 536.4 and a trace of the protonated ion [M + H]⁺ of m/z 537.4, and both ions were summed as the total response for
each carotenoid. The following molecular ions were chosen for the internal standards; *m/z* 277.2 for d₈-α-RP, *m/z* 279.2 for ¹³C₁₀ RP, and *m/z* 546.4 for ¹³C₁₀ β-carotene. For additional identification and monitoring of the retinoids and carotenoids, the diode array detector (DAD) was set at 311 nm for α-retinyl esters, 325 nm for retinyl esters, and 450 nm for the provitamin A carotenoids.

A limitation of our method is the need for an HPLC C₃₀ column with a smaller pore size for UHPLC application. A smaller pore size and shorter column would allow reduced flow rate and analysis time. Reducing the flow rate for UHPLC application can significantly increase the ESI ionization efficiency and thereby further increase the HRMS sensitivity. Even without a column well suited for UHPLC application, the use of highly sensitive HRMS instrumentation allowed us to achieve a lower LOD and LOQ for α- and β-carotene as compared with previously reported methods, which could only use HPLC-DAD. To the best of our knowledge, our HPLC-ESI(+)QTOF-HRMS method is the first to accomplish the simultaneous quantitation of provitamin A carotenoids and their retinyl esters bioconversion products in a single injection.

Other researchers have used LC-MS/MS and LC-MS for quantifying retinyl esters and β-carotene, respectively, which involves two separate injections of the plasma extracts. An LC-ESI-QTOF-HRMS method operated in positive mode was developed and validated for the simultaneous determination of provitamin A carotenoids and their bioconversion products in postprandial TRL samples. Our results showed that an isopropanol-based mobile phase system and 5 mM ammonium formate buffer additive improved the separation and detection of provitamin A carotenoids, α-retinyl esters, and retinyl esters by ESI(+) mass spectrometry. We showed that the here-to-fore limited linear dynamic ranges for these analytes could be extended by using stable isotope-labeled internal standards. This LC-ESI-QTOF-HRMS method can be
applied to the study of carotenoid bioavailability in humans, especially to assess the vitamin A potential of α-carotene containing foods through accurate quantitation of vitamin A without overestimating its vitamin A activity.

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**Author contributions**

The authors’ responsibilities were as follows SDS: implemented and developed the HPLC-ESI-QTOF-HRMS method, implemented the human research participant methods, completed the statistical analyses, and wrote the manuscript; LJS: developed the HPLC-ESI-QTOF-HRMS mobile phase system and provided technical support; JCZ: implemented the cumulative rate ultracentrifugation isolation of plasma triacylglycerol-rich lipoprotein fractions; WST: provided technical support for the development of the quantitative method; WSW: designed and implemented the study, developed the HPLC-ESI-QTOF-HRMS method, analyzed the data, and wrote the manuscript.

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**Conflict of interest**

No author has any conflict of interest.
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**Tables**

**Table 1**

Precision of the target analytes in HPLC-ESI(+)-QTOF-HRMS analyses of the quality control triacylglycerol-rich lipoprotein (TRL) pool isolated from plasma after participants consumed a carrot test meal

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Amount (pmol)</th>
<th>RSD(%)&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Amount (pmol)</th>
<th>RSD(%)&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Retinyl palmitate</td>
<td>3.2 ± 0.4</td>
<td>12.5</td>
<td>4.7 ± 0.7</td>
<td>14.9</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>29.6 ± 2.1</td>
<td>7.1</td>
<td>30.0 ± 1.6</td>
<td>5.3</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>21.1 ± 10.0</td>
<td>47.4</td>
<td>75.7 ± 7.2</td>
<td>9.5</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>69.4 ± 8.7</td>
<td>12.6</td>
<td>51.3 ± 4.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

<sup>1</sup>Values are means ± SDs, n = 6.

<sup>2</sup>Values are means ± SDs, n = 7.

<sup>3</sup>Relative standard deviation, RSD.
**Table 2**

Limits of detection (LOD) and limits of quantitation (LOQ) for the analytes

<table>
<thead>
<tr>
<th>Compounds</th>
<th>LOD</th>
<th>LOQ</th>
<th>Linear dynamic range</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Retinyl palmitate</td>
<td>0.005</td>
<td>0.016</td>
<td>0.064 to 781.0&lt;sup&gt;i&lt;/sup&gt;</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>0.004</td>
<td>0.012</td>
<td>0.035 to &gt; 2,100</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>0.031</td>
<td>0.105</td>
<td>0.043 to 2,125</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.073</td>
<td>0.244</td>
<td>0.338 to 2,079</td>
</tr>
</tbody>
</table>

<sup>i</sup>The 781.0 pmol upper limit of the linear dynamic range for α-retinyl palmitate, a more minor plasma metabolite, was the highest amount on-column that was evaluated.
Table 3

Intra- and inter-day precision of the calibration standards

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Intra-day</th>
<th>Inter-day</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Amount (pmol)</td>
<td>RSD(%)</td>
</tr>
<tr>
<td>α-Retinyl palmitate</td>
<td>18.6 ± 0.8</td>
<td>4.3</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>19.9 ± 0.2</td>
<td>1.0</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>73.6 ± 5.2</td>
<td>7.1</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>63.9 ± 0.9</td>
<td>1.4</td>
</tr>
</tbody>
</table>

1Values are means ± SDs, n = 7.

2Values are means ± SDs, n = 7 for each of 3 days.

3Relative standard deviation, RSD.
Table 4

Inter-day precision of postprandial triacylglycerol-rich lipoprotein absorption curves of all target analytes after research participants consumed a cooked carrot test meal

<table>
<thead>
<tr>
<th>Analyte</th>
<th>AUC †</th>
<th>RSD(%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>nmol·h/L plasma</td>
<td></td>
</tr>
<tr>
<td>α-Retinyl palmitate</td>
<td>4.7 ± 0.6</td>
<td>12.8</td>
</tr>
<tr>
<td>Retinyl palmitate</td>
<td>25.7 ± 2.6</td>
<td>10.1</td>
</tr>
<tr>
<td>α-Carotene</td>
<td>107.9 ± 10.6</td>
<td>9.8</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>113.6 ± 9.1</td>
<td>8.0</td>
</tr>
</tbody>
</table>

† Area under the curve (AUC). Values are means ± SDs, n = 3 replicate absorption curves
Figure 1. An overlay of the system pressure readings of the HPLC-ESI(+)–QTOF–HRMS system, incorporating the column back pressure, during the analysis of identical extracts of a postprandial triacylglycerol-rich lipoprotein (TRL) fraction. Shown are an MTBE-based non-ESI compatible mobile phase system gradient (black) and an isopropanol-based ESI-compatible system gradient (blue) analyzed on a C$_{30}$ Carotenoid Column, 4.6 mm × 250 mm, 3 µm particle size.
**Figure 2.** Overlay of the total ion current (TIC) chromatograms of two injections from an extract of a postprandial plasma triacylglycerol-rich lipoprotein (TRL) fraction analyzed by HPLC-ESI(+)-QTOF-HRMS. Shown is an MTBE-based non-ESI-compatible mobile phase system (black) compared with the isopropanol-based ESI-compatible mobile phase system (blue).
Figure 3. Extracted ion chromatograms (EICs) of retinyl esters at $m/z$ 269.2 in two injections of an extract of a postprandial plasma triacylglycerol-rich lipoprotein (TRL) fraction analyzed by HPLC-ESI(+)–QTOF-HRMS. Shown is an MTBE-based non-ESI-compatible mobile phase system (black) compared with the isopropanol-based ESI-compatible mobile phase system (blue). $\alpha$-RO, $\alpha$-retinyl oleate; RO, retinyl oleate; $\alpha$-RP, $\alpha$-retinyl palmitate; RP, retinyl palmitate; RS, retinyl stearate.
**Figure 4.** Extracted ion chromatograms (EICs) of $\alpha$- and $\beta$-carotene at $m/z$ 536.4+537.4 in two injections from an extract of a postprandial plasma triacylglycerol-rich lipoprotein (TRL) fraction analyzed by HPLC-ESI(+)-QTOF-HRMS. Shown is an MTBE-based non-ESI-compatible mobile phase system (black) compared with the isopropanol-based ESI-compatible mobile phase system (blue). $\alpha$C, $\alpha$-carotene; $\beta$C, $\beta$-carotene.
**Figure 5.** Effects of different mobile phase buffer additives on the analyte signal responses by HPLC-ESI(+)-QTOF-HRMS. Shown are the responses of α-retinyl palmitate, retinyl palmitate, and β-carotene (mean ± SDs), which were spiked into a plasma triacylglycerol fraction, which was then extracted and analyzed with an isopropanol-based mobile phase system containing 0.1 % formic acid, 5 mM ammonium formate plus 0.1 % formic acid, or 5 mM ammonium formate. One-way ANOVA followed by the Student’s t-test was used to compare the effects of the different buffer additives on the signal response. * $P < 0.05$, ** $P < 0.01$
Figure 6. Internal standard calibration curves for $\alpha$-carotene, $\beta$-carotene, $\alpha$-retinyl palmitate, and retinyl palmitate showing the linear responses. Shown are the ratios of the peak areas for the analyte and internal standard plotted against the injected amount of the analyte. $\alpha$C, $\alpha$-carotene; 13C10-$\beta$C, $^{13}$C-labeled $\beta$-carotene internal standard; $\beta$C, $\beta$-carotene; $\alpha$-RP, $\alpha$-retinyl palmitate; $d_8$-$\alpha$-RP, deuterium-labeled ($d_8$) $\alpha$-retinyl palmitate internal standard; RP, retinyl palmitate; 13C10-RP, $^{13}$C-labeled retinyl palmitate internal standard retinyl palmitate internal standard.
Figure 7. Overlay of the extracted ion chromatograms (EICs) for the analytes in an extract of a plasma triacylglycerol rich (TRL) fraction after participants consumed a test meal of cooked carrots. The traces shown are $m/z$ 269.2 (dark blue) for retinyl esters and their $\alpha$-analogues; $m/z$ 277.2 (turquoise) for the d$_8$-$\alpha$-retinyl palmitate ($\alpha$-RP-d$_8$) internal standard; $m/z$ 279.2 (purple) for the $^{13}$C$_{10}$-retinyl palmitate ($^{13}$C 10-RP) internal standard; $m/z$ 536.4+537.4 (pink) for $\alpha$- and $\beta$-carotene; $m/z$ 546.4 (black) for the $^{13}$C$_{10}$ $\beta$-carotene ($^{13}$C10-$\beta$C) internal standard.
Figure 8. Inter-day precision of replicate HPLC−MS analyses of extracts of the plasma TRL fraction isolated after participants consumed a model test meal (carrots). At each time point, plasma samples were pooled across participants (n = 2) for the isolation of the TRL fraction. Analyte contents in the plasma TRL fractions are presented as means ± SEMs, n = 3 replicate analyses. TRL, triacylglycerol-rich lipoprotein.
CHAPTER 4. VITAMIN A EQUIVALENCE OF THE PROVITAMIN A CAROTENOIDS IN BIOFORTIFIED BANANA FRUIT

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Modified from a manuscript in preparation for The American Journal of Clinical Nutrition

Abstract

\textbf{Background:} Bananas with provitamin A contents enhanced through biotechnology are a sustainable food-based intervention with the potential to alleviate vitamin A deficiency in East-Central Africa where diets rely upon bananas as the major dietary staple.

\textbf{Objective:} Our objective was to separate $\alpha$-retinyl palmitate from retinyl palmitate and accurately quantify the vitamin A activity of biofortified cooked banana fruit.

\textbf{Design:} Twelve healthy women each consumed three 200-g portions of cooked banana fruit as follows: 1) transgenic biofortified banana fruit containing 1415.3 µg (2.64 µmol) of $\beta$-carotene equivalents, 2) wild-type banana fruit with a $\beta$-carotene reference dose containing 536.8 µg (1.00 µmol) added $\beta$-carotene, and 3) wild-type banana fruit with a vitamin A reference dose containing 262.5 µg RAE (0.92 µmol) added retinyl palmitate. The test meals contained 8.0 g added peanut oil and were consumed in random order separated by ≥ 2 wk. Blood samples were collected at selected time points over 9 h. The $\alpha$-retinyl palmitate, retinyl palmitate, $\alpha$- and $\beta$-carotene were extracted from plasma triacylglycerol–rich lipoprotein (TRL) fractions (chylomicron and large VLDL) separated by cumulative-rate ultracentrifugation. The analytes were quantified by using HPLC–quadrupole time-of-flight–mass spectrometry. The trial was
registered at clinicaltrials.gov as NCT02702622.

**Results:** Mean (± SD) areas under the curve for retinyl palmitate in the TRL fractions (nmol·h) were 63.0 ± 50.3, 167.3 ± 119.6, and 167.8 ± 116.4 after consumption of the provitamin A-biofortified banana fruit, the wild-type banana fruit with the β-carotene reference dose, and the wild-type banana fruit with the vitamin A reference dose, respectively. The vitamin A equivalence values of the provitamin A carotenoids in the biofortified banana fruit and the β-carotene reference dose added to the wild-type banana fruit were 18.52 ± 10.8 µg (mean ± SD) and 2.29 ± 0.93 µg, respectively.

**Conclusion:** The provitamin A carotenoids in the biofortified Cavendish banana fruit had a bioconversion efficiency similar to that reported for carotenoid-rich vegetables, such as carrots or spinach, but less than that reported for starchy matrices such as cassava, maize, and rice.

**KEY WORDS:** α-carotene, bioconversion, biofortification, vitamin A deficiency, vitamin A equivalence

**Introduction**

Vitamin A deficiency remains prevalent in South Asia and sub-Saharan Africa and affects primarily preschool-aged children and pregnant women (1). The persistent high prevalence of vitamin A deficiency in these regions despite vitamin A supplementation programs has led to a call for food-based interventions, which increase the daily dietary intake of vitamin A (2). The public health efficacy of high-dose vitamin A supplementation is well established in children younger than 5 years in terms of reducing mortality and nutritional blindness (3). However, the resulting transient effect on serum retinol highlights the importance of a sustainable food-based approach as a complementary intervention to prevent vitamin A deficiency (3,4). Food-based interventions such as provitamin A biofortified crops can
sustainably improve the vitamin A status of at-risk populations (3,4). The food-based solutions under development include genetically-modified crops enriched in provitamin A carotenoids, which offer the advantages of low cost, sustainability, and potential for accessibility by poor populations in rural areas (4).

Bananas are a leading dietary staple and cash crop in some low-income, food-deficit countries (5). Among the African countries, Uganda, Rwanda and Cameroon are the main consumers of bananas (including plantains and non-Cavendish cultivars that are low in micronutrients) with a reported yearly per capita consumption of 200 kg (5). Domestication of banana fruit produces edible banana cultivars that are near-sterile triploids, making conventional breeding challenging for crop improvement (6). As a “proof-of-concept”, researchers at Queensland University of Technology (QUT) and the National Agricultural Research Organization (NARO) Uganda have successfully developed a provitamin A enriched “Cavendish” cultivar through the expression of a Fe’i banana-derived phytoene synthases 2a cis-gene (6,7). This genetic modification technique enabled the enrichment of the near-sterile triploid banana fruit with provitamin A α- and β-carotene.

The asymmetric provitamin A carotenoid, α-carotene, is cleaved in the intestinal mucosa to produce one molecule of retinol and its biologically inactive analogue, α-retinol. Recently, Goetz et al. (8) emphasized the importance of separating the derivatives of retinol and α-retinol during analysis to avoid overestimation of the vitamin A activity of α-carotene-containing foods. Subsequently, Cooperstone et al. (9) reported high inter-individual variability in the efficiency of metabolizing α-carotene to vitamin A after consuming carrots, which are a rich source of α-carotene. The direct assessment of the α-carotene contribution requires quantitation of the postprandial α-retinol derivatives, i.e., α-retinyl esters.
An important step in the development of the biofortified bananas is to evaluate their bioefficacy as a source of vitamin A (10). To evaluate bioefficacy, our objective is to accurately quantify the vitamin A equivalence – i.e., the quantity of β-carotene equivalents in the fruit that will provide vitamin A activity equivalent to 1 μg of consumed retinol. HPLC-quadrupole time-of-flight (HPLC-QTOF) mass spectrometry was used to quantify the newly absorbed retinyl palmitate, as well as the α-retinyl palmitate product of α-carotene bioconversion for each participant.

Subjects and methods

Participants

Twelve, non-smoking, healthy women, ages 18-40 y, were enrolled in the study from April to June 2016 at the Iowa State University Nutrition and Wellness Research Center. The mean (± SD) age was 23.0 ± 4.3 y; the mean (± SD) body mass index (BMI; in kg/m²) was 23.5 ± 2.0. Figure 1 shows the CONSORT flow diagram for the recruitment procedures. After providing written informed consent, each participant was interviewed by using a standardized questionnaire which assessed health and lifestyle factors. Each participant’s body weight and height were measured. Those who appeared to qualify for participation were invited to donate a fasting blood sample for additional health screening, which included a blood biochemistry profile, blood lipid panel, and complete blood count. The inclusion criteria included good health, as demonstrated by the standardized interview, plasma biochemistry profile, plasma thyroid-stimulating hormone (TSH) level, normolipidemia, and BMI < 28. Abnormal TSH level reflects thyroid hormone function that may affect provitamin A conversion to vitamin A (11). Exclusion criteria were current or recent (previous 12 mo) cigarette smoking, frequent consumption of alcoholic beverages (>1 drink/d), current or recent (previous 1 mo) use of dietary supplements,
current or recent (previous 6 mo) use of hormonal contraceptives, and current or recent (previous 1 mo) use of medications known to affect lipid metabolism. Also excluded were those with a history of restrictive eating, those with lactose intolerance, and vegetarians. The study protocol was approved by the Iowa State University Institutional Review Board.

Dietary protocols

Each participant completed three 7 d study periods in randomized order and separated by \( \geq 2 \) wk. During each study period, the participants consumed one of three 200-g cooked banana portions, as follows: 1) cooked transgenic fruit containing 1,415 µg (2.6 µmol) \( \beta \)-carotene equivalents with 8.0 g added peanut oil, 2) cooked wild-type fruit containing a trace amount of \( \beta \)-carotene equivalents (0.13 µg or 0.25 µmol) with 536.8 µg (1.00 µmol) \( \beta \)-carotene added in 8.0 g of peanut oil, and 3) cooked wild-type fruit containing a trace amount of \( \beta \)-carotene equivalents (0.13 µg or 0.25 µmol) with 262.5 µg RAE (0.92 µmol) vitamin A palmitate added in 8.0 g of peanut oil.

Participants were instructed to adhere to the following dietary protocol during each study period; all meals provided were standardized, pre-weighed, conventional foods: On days 1-3, the participants were asked to avoid carotenoid-rich foods, as listed on an instruction sheet. On days 4-6, the participants consumed standardized, weighed meals, including breakfast, lunch, dinner, and snacks. Breakfast and dinner were consumed on site at the Iowa State University Nutrition and Wellness Research Center under supervision. Lunch and snacks were carried out and consumed outside the research center. The nutrient contents in the standardized diet were analyzed by Nutritionist Pro nutrition analysis software (Axxya Systems, Stafford, TX). The daily diet provided 9.0 MJ, 71 g protein, 42 g fat, and 372 g carbohydrate, 56 µg \( \beta \)-carotene, 0 µg \( \alpha \)-carotene and 86 µg RAE of vitamin A. On day 7, after an overnight (12-h) fast, each
participant consumed the 200-g portion of cooked banana fruit. After consuming the banana portion, the participants consumed a carotenoid- and vitamin A-free snack (at 3.5 h) and lunch (at 5 h). The snack and lunch contained 0.3 g and 1.6 g fat, respectively. The calories in the snack and lunch were 103 and 283 kcal, respectively.

**Banana portions**

The transgenic biofortified and wild-type Cavendish bananas were grown at the Centre for Tropical Crops and Biocommodities, Queensland University of Technology (QUT), Brisbane, Australia. The fresh fruit was shipped via air freight and arrived at Iowa State University within 36 h of shipment. Ethylene-absorbing packets and brown paper bags were used to achieve uniform ripening. A 4-g slice from the center of each ripened finger was retained to produce composited transgenic and wild-type fruit for carotenoid and proximate analyses. The remaining fruit was cooked 3-5 min in boiling water until softened. The cooked fruit was drained, cooled to room temperature, and mashed for 7 sec in a food processor. Aliquots of the composited cooked, mashed fruit were saved for future carotenoid analysis; the remainder was immediately aliquoted into 200-g portions for eventual consumption by the study participants. The fresh fruit slices and the portions of the boiled, mashed fruit were stored at −80°C in an ultralow freezer dedicated for food use.

**Carotenoid and proximate analyses of the bananas**

The carotenoids in the fresh and cooked banana fruit were extracted and analyzed in triplicate \((n = 3)\) according to a modification of the method by Muzhingi et al. (12). Briefly, 3 g of homogenized banana puree was transferred to a 50-mL test tube. Ten mL ethanol containing 1 g/L butylated hydroxytoluene (BHT) and 7500 ng internal standard \((\gamma\text{-carotene})\) were added. \(\gamma\)-Carotene was used as the internal standard to avoid coelution with phytofluene and provide
accurate quantitation of all carotenoid analytes in the banana fruit. The sample mixture was vortexed for 2 min and centrifuged at 700 g × 5 min. The ethanol-containing supernatant was transferred to a 50-mL volumetric flask and the extraction was repeated four times with 10 mL of tetrahydrofuran (THF) containing 1 g/L BHT. The THF layers were combined with the ethanol layer and the final volume was brought to 50 mL. One mL of the extract was dried under vacuum and reconstituted with 400 µL methyl-tert-butyl-ether (MTBE): methanol (1:3, by vol). A 100 µL aliquot was injected into the HPLC system, which included a 717Plus autosampler with the temperature control set at 5 °C, two 515 solvent delivery systems, and a 2996 photodiode array detector (Waters Corporation, Milford, MA). The carotenoids were separated on a 5 µm C30 Carotenoid Column (4.6 × 250 mm, YMC, Inc) at a flow rate of 1.0 mL/min. The solvents contained methanol: aqueous ammonium acetate (13 mM) 96.5:3.5 (by vol) (mobile phase A) and methanol:MTBE:water 40:58:2 (by vol) (mobile phase B). The following gradient was used: 0-15 min, linear gradient from 0% to 45% solvent B; 15-35 min, linear gradient from 45% to 55% solvent B; 35-45 min, linear gradient from 55% to 85% solvent B; 45-65 min, linear gradient from 85% to 100% solvent B. All solvents were HPLC grade and were purchased from Fisher Scientific (Fairlawn, NJ).

Reference doses

The β-carotene reference dose was prepared from a microcrystalline suspension of β-carotene in corn oil (β-Carotene 30% FS, product code 04 27233 004; DSM Nutritional Products Inc, Parsippany, NJ). The vitamin A reference dose was prepared from a concentrate in corn oil without stabilizers (Vitamin A Palmitate, 1.7 million IU/g, product code 04 24005 004; Roche Vitamins, Parsippany, NJ). Each concentrate was warmed briefly to 40°C in a water bath and inverted to mix. The concentrations of the reference dose were confirmed by HPLC analysis, as
described above. To provide the reference doses, 8.0 g peanut oil containing 536.8 μg of β-carotene or 262.5 μg RAE retinyl palmitate was added to the wild-type banana portion. A corresponding 8.0 g of unfortified peanut oil was added to the biofortified banana portion.

**Isolation of triacylglycerol-rich-lipoprotein fractions**

Blood samples (10 mL) were collected from a forearm vein and transferred to evacuated blood collection tubes containing dipotassium EDTA (13). A baseline blood sample was collected after a 12-h overnight fast and immediately preceding consumption of the fruit portion. Additional blood samples were collected postprandially at 2, 3.5, 5, 7, and 9 h. The chylomicron and large VLDL (VLDLa) fractions were isolated from each plasma sample by cumulative rate ultracentrifugation (13) and stored at -80°C until analyzed. Quality control (QC) materials were aliquots from a combined plasma chylomicron and VLDLa pool. The composite pool was prepared using TRL fractions isolated at a selected time point collected after participants consumed standardized carrot meals in order to produce α-retinyl palmitate-containing QC materials. The protocol used to prepare the quality control material was a separate protocol, which was also approved by the Iowa State University Institutional Review Board.

**HPLC-quadrupole time-of-flight analysis of the postprandial lipoprotein fractions**

The isolated chylomicron and VLDLa fractions were combined at each time point and 680 µL of the combined fraction was extracted by deproteination with 200 proof ethyl-alcohol. Internal standards were added as follows: 6 ng d₈-α-retinyl palmitate, 12 ng ¹³C₁₀-retinyl palmitate, and 6 ng ¹³C₁₀-β-carotene. In addition, 3.6 ng of unlabeled epsilon-carotene was added to monitor the performance of the instrumentation. The analytes were extracted three times with hexane (1 g butylated hydroxytoluene/L). The hexane layers were combined and dried under vacuum. The dried extract was reconstituted with 60 µL methyl-tert-butyl ether:
methanol (1:1; vol: vol). A 50-μL aliquot was injected into the HPLC-QTOF system. The α-retinyl esters, retinyl esters, α- and β-carotene were separated by using a 3 μm C30 Carotenoid Column (4.6 × 250 mm) (YMC Inc) protected with a 3 μm C30 guard column (YMC Inc). The solvents used to wash the analytical column included solvent A1: isopropanol/acetonitrile/MTBE (50:25:25) (by vol) and solvent B1: MTBE/isopropanol/water (80:17:3) (by vol). The acquisition solvents included solvent A2: methanol/acetonitrile/water with 5 mM ammonium formate (65:25:10) (by vol) and solvent B2: isopropanol/acetonitrile/MTBE with 5 mM ammonium formate (50:25:25) (by vol). The acquisition gradient was as follows: 0-9 min, linear gradient 59% to 62% solvent B2; 9-11 min, linear gradient 62% to 70% solvent B2; 11-20 min, hold at 70% solvent B2. The components and parameter settings of the HPLC-QTOF system were specified previously (Chapter 3). During positive ion electrospray, both the α-retinyl esters and retinyl esters produced an abundant in-source dehydrated retinol fragment ion base peak at m/z 269.2 [MH - H₂O]⁺, which was used for quantitation (14). For the carotenoids, positive ion electrospray produced an unfragmented dominant molecular ion [M]⁺ of m/z 536.4 and a trace of the protonated ion [M + H]⁺ of m/z 537.4; both ions were summed to quantify the response for each carotenoid (15).

Data analyses

The postprandial plasma TRL responses for α-retinyl palmitate, retinyl palmitate, α- and β-carotene in the combined chylomicron and VLDLₐ fractions were calculated as contents isolated from 1 L plasma (nmol/L plasma) and presented as the content (nmol) in the entire plasma pool based upon the calculated plasma volume for each participant (0.0427 L × kg body wt) (13,16). The postprandial response curves were plotted as the TRL content change (nmol) as the y-axis and time (h) as the x-axis. The 0–9-h area under the curve (AUC) (nmol·h) for each
analyte was calculated from the postprandial response curve by baseline-corrected trapezoidal approximation (13). All statistical analyses were conducted using RStudio version 1.3.1093 (RStudio, Public Benefit Corporation).

The vitamin A equivalence values for the provitamin A-biofortified banana fruit were calculated as described previously (13,16) and based on the proportional comparison of the vitamin A formed (nmol) after consumption of the biofortified fruit and the vitamin A formed (nmol) after consumption of the wild-type fruit with vitamin A reference dose.

Vitamin A (nmol) formed from the β-carotene equivalents in the biofortified banana fruit

\[
\text{Vitamin A (nmol)} = \frac{\text{Retinyl palmitate AUC}}{\text{Retinyl palmitate AUC}} \times 916.4 \text{ nmol} \quad (1)
\]

Where 916.4 was the vitamin A reference dose (in nmol).

Vitamin A equivalence (by wt) = \(\frac{\beta\text{-Carotene equivalents in the biofortified banana (nmol)} }{\text{Vitamin A formed from the } \beta\text{-carotene equivalents (nmol)} } \times 536.8 \quad (2)\)

where 536.8 and 286.5 are the molecular weight of β-carotene and retinol, respectively. The β-carotene equivalents in the biofortified banana fruit were calculated as trans-β-carotene plus 0.5 \(\times (\text{cis-β-carotene isomers plus trans- } \alpha\text{-carotene}) + 0.25 \times \text{cis-α-carotene (by wt)} \) (17).

Additional calculations (see Appendix) were applied to correct for the minor amounts of α- and β-carotene present in the wild-type banana fruit.

Results

Analysis of the banana fruit

The provitamin A carotenoid contents in the cooked banana fruit are shown in TABLE 1. The biofortified banana carotenoid profile analyzed by using HPLC with photodiode array detection is shown in Figure 2. The major provitamin A carotenoid in the biofortified cooked banana fruit was trans-α-carotene followed by trans-β-carotene, cis-α-carotene and cis-β-
carotene; the ratio of the trans-α-carotene to trans-β-carotene was 1.60 (by wt). The biofortified banana fruit also contained a significant amount of the non-provitamin A carotenoid, lutein. The provitamin A carotenoid contents in the biofortified cooked banana fruit portions were highly reproducible across analyses. The CVs for trans-α-carotene, trans-β-carotene, cis-α-carotene, and cis-β-carotene, were 1.56%, 1.07%, 5.6% and 4.6%. The wild-type fresh banana fruit contained trace amounts of trans α-carotene, trans β-carotene and non-provitamin A lutein. The β-carotene equivalents in the 200-g of the cooked fruit were 128.7 ± 3.5 µg β-carotene equivalents for wild-type fruit and 1,415.3 ± 8.3 µg β-carotene equivalents for biofortified fruit.

In our cooking protocol, the retention of provitamin A carotenoids were over 100% after boiling in both wild-type and biofortified banana fruit. The retention exceeding 100% may be attributed to the change of soluble solids in the fruit during boiling. A previous report observed that a 15 % loss of soluble solids in sweet potato during blanching was associated with a significant increase in β-carotene content (18). The moisture contents of the cooked fruit were 79.8 ± 0.0% and 77.4 ± 0.1% for biofortified and wild-type, respectively. The moisture contents in the peel and pulp change during fruit ripening (19). Through careful control of the ripening conditions, we were able to achieve comparable moisture contents in the biofortified and wild-type banana fruit.

**Appearance of α-retinyl palmitate, retinyl palmitate, α- and β-carotene in the postprandial plasma triacylglycerol-rich lipoprotein fractions**

For each participant, the use of HPLC-QTOF enabled measurement of the newly absorbed α-carotene, β-carotene, the major retinyl palmitate bioconversion product, and the α-retinyl palmitate cleavage product of α-carotene after the consumption of the biofortified banana fruit. A representative HPLC-QTOF extracted ion chromatogram (EIC) of an extract of a plasma combined TRL fraction at 3.5 h (C_{max}) is shown in **Figure 3**. Mean baseline-corrected
postprandial plasma TRL α-retinyl palmitate and retinyl palmitate response curves are shown in **Figure 4**. For each participant, the AUC values for each of the banana portion standardized per µmol ingested β-carotene or vitamin A are listed in **TABLE 2**. The vitamin A equivalence calculated for provitamin A biofortified banana fruit and β-carotene reference dose consumed with wild-type banana fruit were 18.52 ± 3.12 and 2.29 ± 0.27, respectively (**TABLE 3**). These mean vitamin A equivalence values were significantly different by paired t test (P < 0.001).

An aliquot of the plasma TRL quality control pool was analyzed with each sample set. The inter-day precision was expressed as percent relative standard deviation (% RSD) of the HPLC-QTOF analyses of α-retinyl palmitate, retinyl palmitate, α- and β-carotene in the QC material from 36 separate analytical runs (**TABLE 4**). The % RSD values for these analytes were 10.1%, 4.9%, 13.8%, and 7.4%, respectively. These % RSD values are well within the acceptable range for targeted quantitative bioanalysis (20).

**Discussion**

The transgenic provitamin A carotenoid-enriched Cavendish banana fruit used in the present study was produced by the insertion of the gene for phytoene synthase, which was isolated from the Fe’i banana cultivar Asupina and under the control of a maize polyubiquitin promoter (6). Phytoene synthase is a key enzyme that catalyzes the rate-limiting step in carotenoid biosynthesis (21). We provided an accurate quantitative analysis of the vitamin A equivalence of the α- and β-carotene in the biofortified banana fruit by excluding the α-retinyl palmitate derivative of the inactive α-retinol metabolite of the α-carotene (9). This is the first study in humans that addresses the bioefficacy of the provitamin A carotenoids in the biofortified banana fruit as a source of vitamin A.
Our analysis showed that on average, 18.5 µg of the β-carotene equivalents in biofortified Cavendish banana fruit is needed to provide 1 µg of retinol (**TABLE 3**). The provitamin A carotenoids in the biofortified Cavendish banana fruit had a bioconversion efficiency similar to that reported for carotenoid-rich vegetables. In healthy adults who were fed deuterated vegetables, 14.8 µg of deuterated carrots and 20.9 µg of deuterated spinach were required to provide 1 µg of retinol (16). The bioconversion efficiency in the biofortified bananas is lower compared with the bioavailability of β-carotene in biofortified starchy matrices. With a similar experimental approach employing the consumption of a single serving (250 g) of β-carotene-biofortified maize porridge, we previously showed that 6.48 ± 3.51 µg (mean ± SD) of the β-carotene in the maize were required to provide 1 µg of retinol (13). The vitamin A equivalence of Golden Rice was reported to be 3.8 ± 1.7 to 1 in healthy adults (22). The repeated consumption of starchy sweet potato, however, provided a vitamin A equivalence of 13.1 µg to 1 in healthy Bangladeshi men (23).

We utilized the plasma TRL fraction (chylomicrons plus VLDL<sub>A</sub>) as an established vehicle to isolate the newly absorbed provitamin A carotenoids and intestinally-derived retinyl palmitate (24–27). This approach allows the differentiation of carotenoids and retinyl esters of endogenous origin from newly absorbed provitamin A carotenoids and their intestinal cleavage product retinyl esters (28). We have validated our experimental approach previously by including a reference dose treatment consisting of a known amount of pure β-carotene dissolved in oil (13). In the present study, we again validated our approach; the vitamin A equivalence of the β-carotene reference dose was 2.29 ± 0.93, which is in agreement with the value reported by the U.S. National Academy of Medicine (29).
In the present study, \(\alpha\)-carotene was the dominant provitamin A carotenoid in the biofortified Cavendish banana fruit fed to the participants. Recently, the large interindividual variability in \(\alpha\)-carotene bioconversion efficiency was reported using an analytical approach that excludes \(\alpha\)-retinol in the estimation of the vitamin A activity of \(\alpha\)-carotene-containing foods (8,9). Estimation of vitamin A activity of \(\alpha\)-carotene in \(\alpha\)-carotene-containing foods by the traditional analytical approach that did not resolve \(\alpha\)-retinol likely overestimated the true bioconversion efficiency. Due to the minute amounts of postprandial provitamin A carotenoids and their retinyl ester bioconversion products in the isolated plasma TRL fractions, the contribution of \(\alpha\)-carotene in the biofortified bananas to the postprandial vitamin A requires highly selective and sensitive instrumentation for accurate vitamin A equivalence determination. The HPLC-QTOF method we developed (Chapter 3) demonstrated enhanced selectivity and sensitivity to quantify the postprandial TRL appearance of \(\alpha\)-carotene, \(\beta\)-carotene, and their retinyl ester bioconversion products, as well as the \(\alpha\)-retinyl palmitate product of \(\alpha\)-carotene. We chose to use the stable isotope-labeled analogs of the analytes as internal standards to provide accurate quantitation of the analytes. Selecting stable isotope-labeled analogs that coelute with their respective analyte allows accurate LC-MS quantitative analysis, as it best corrects for background interference (30).

The absorption kinetics of \(\alpha\)-retinyl palmitate and retinyl palmitate in the current study showed similar trends with those reported by Cooperstone et al (9). The incorporation of \(\alpha\)-retinyl palmitate into plasma TRL was similar to that of retinyl palmitate, as indicated by the shape of the postprandial \(\alpha\)-retinyl palmitate and retinyl palmitate response curves for each participant. The times (\(T_{\text{max}}\)) for the maximum contents (\(C_{\text{max}}\)) of \(\alpha\)-retinyl palmitate and retinyl palmitate (TABLE 5) in the plasma TRL fraction were similar across most of the participants.
except for one participant. Two out of the twelve participants have a kinetic profile showing a continued elevated retinyl palmitate content after $C_{\text{max}}$ that persisted until the 7 h time point. The elevated retinyl palmitate level in these participants may be explained by a delayed clearance of retinyl palmitate (31,32).

Our results showed that although more $\beta$-carotene equivalents were provided in the biofortified bananas (1,415 µg) compared with the $\beta$-carotene reference dose (536.8 µg), the vitamin A equivalence value after consuming biofortified banana is 8-fold higher than that of $\beta$-carotene reference dose. When the amount of $\beta$-carotene consumed was standardized, on average, the participants formed 7.3-fold more retinyl palmitate after the consumption of $\beta$-carotene reference dose (Table 3). The utilization of provitamin A carotenoids during absorption is substantially affected by the complex plant matrix in which they are embedded. Dietary fiber in banana fruit, such as resistant starch in green bananas or soluble fiber pectin in ripe fruit, is a limiting factor in the absorption of carotenoids because it interferes with bile acid micellarization and prevents absorption of lipids (33). Green bananas contain considerably more resistant starch; this starch converts to soluble sugars as the fruit ripens in a cultivar-dependent manner (34,35). Dietary pectin is a soluble polysaccharide fiber found in the cell wall in plant tissues. As the fruit ripens, the pectin in the cell wall solubilizes and water-soluble pectin increases (36). Pectin has been shown to reduce the bioavailability of $\beta$-carotene in humans (37,38). In a Mongolian gerbil model, the effects of ripeness and thermal processing on the bioefficacy of provitamin A carotenoids in a variety of banana cultivars were studied (39). The investigators concluded that the ripening of the fruit did not improve the provitamin A bioefficacy, which may be explained by increasing pectin presence in the fruit. However, the pectin content in the fruit was not reported. They concluded that in the animal model, thermal
processing improved the bioavailability of the provitamin A carotenoids in the bananas’

Another possible explanation of the low bioavailability of the provitamin A in
biofortified bananas may be attributed to the conversion efficiency of the predominant
provitamin A α-carotene. The provitamin A conversion efficiency is highly variable between
individuals (9,40). The single nucleotide polymorphisms (SNPs) associated with the β-carotene-
15, 15′- oxygenase (BCO1) gene, which encodes for the cytosolic cleavage enzyme for
provitamin A carotenoids, may influence the conversion of provitamin A to retinal (41).
Although we did not measure the genetic variants in this study, we observed that five
participants, who were also Caucasian, had an equivalent or higher AUC value for α-retinyl
palmitate than retinyl palmitate (ratio retinyl palmitate: α-retinyl palmitate), indicating high
conversion efficiency of α-carotene to vitamin A. Two single nucleotide polymorphisms (SNPs)
in the human BCO1 gene have been identified that affect the enzyme catalytic activity and
individuals with these SNPs were categorized as poor converters for β-carotene contributing to
lower TRL retinyl palmitate:β-carotene ratios (41). Recently, a combination of SNPs on 12
genes were associated with the intestinal conversion efficiency of β-carotene (42). However,
there is a lack of evidence regarding the association of genetic variants with the efficiency of the
intestinal uptake of α-carotene.

Consuming a provitamin A-enriched staple crop is a sustainable approach to increase the
daily intake of vitamin A (2). The initial target level of provitamin A in the biofortified bananas
was 20 µg β-carotene equivalents per g fresh weight (43). This target was intended to meet 50%
of the Estimated Average Requirement (EAR) for vitamin A for the vulnerable populations
based on 1) estimated retinol equivalency of 12:1, 2) estimated provitamin A loss during cooking
and processing of 50%, and 3) average intake of bananas/plantains in East-Central African
countries of 300 g/day for adult women and 150 g/day for children age 3-5 y (43). The cooked biofortified banana fruit in our controlled human study contained 7.1 µg β-carotene equivalents per g, which is 2.8 times lower than the target level. Based on the results of our study, consuming 200 g of provitamin A banana fruit would provide 76 µg RAE or 15% of the EAR (500 µg RAE/day) for an adult woman (29). A 300-g serving size would meet 23% of the EAR for an adult woman. For children age 3-5 y, 150 g of provitamin A banana fruit would provide 57.3 µg RAE or 20% of the EAR (275 µg RAE/day). Banana breeders continue to improve the biofortified bananas with the goal to deliver a provitamin A biofortified line that would meet 50% of the EAR of the target populations (6). In conclusion, the biofortified bananas show considerable promise to effectively contribute to the prevention of endemic vitamin A deficiency in East Central Africa.

Acknowledgments

We thank Diane Gillott for implementing the phlebotomy protocols and our study participants for their dedication.

The authors’ responsibilities were as follows – SDS: implemented the study, developed the method used for the HPLC-HRMS analyses; analyzed the data, and wrote the manuscript; HY: implemented the study; RH: developed and produced the biofortified bananas for the study; and WSW: designed and implemented the study, developed the method used for the HPLC-HRMS analyses, analyzed the data, and wrote the manuscript.

References


17. Sweeney JP, Marsh AC. Liver Storage of vitamin A in rats fed carotene stereoisomers. J
110


### TABLE 1

Provitamin A carotenoid contents in the banana fruit portions

<table>
<thead>
<tr>
<th>Components</th>
<th>Biofortified</th>
<th>Wild-type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µg/200 g</td>
<td></td>
</tr>
<tr>
<td>Lutein</td>
<td>306.5 ± 0.6</td>
<td>184.4 ± 4.8</td>
</tr>
<tr>
<td>Phytoene</td>
<td>371.2 ± 16.8</td>
<td>ND²</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>84.1 ± 4.3</td>
<td>ND</td>
</tr>
<tr>
<td>Total cis-α-carotene</td>
<td>176.8 ± 9.9</td>
<td>30.9 ± 1.6</td>
</tr>
<tr>
<td>Total cis-β-carotene</td>
<td>143.0 ± 6.6</td>
<td>12.1 ± 1.3</td>
</tr>
<tr>
<td>Trans α-carotene</td>
<td>1156.3 ± 18.0</td>
<td>104.5 ± 3.1</td>
</tr>
<tr>
<td>Trans β-carotene</td>
<td>721.5 ± 7.7</td>
<td>62.7 ± 1.2</td>
</tr>
<tr>
<td>β-Carotene equivalents³</td>
<td>1415.3 ± 8.3</td>
<td>128.7 ± 3.5</td>
</tr>
</tbody>
</table>

¹All values are means ± SDs.  n = 3.

²ND, not detected.

³β-Carotene equivalents were calculated on the basis that trans α-carotene and cis-β-carotene have 50% of the provitamin A activity of trans β-carotene (44), and cis-α-carotene has 25% of the provitamin A activity of trans β-carotene (17).
**TABLE 2**

Area under the response curve (AUC) 0–9-h values for the appearance of retinyl palmitate in the plasma triacylglycerol–rich lipoprotein fraction after consumption of the banana portions

<table>
<thead>
<tr>
<th>Participant</th>
<th>Wild-type with βC reference dose</th>
<th>Biofortified</th>
<th>Wild-type with vitamin A reference dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>88.6&lt;sup&gt;1&lt;/sup&gt;</td>
<td>8.4&lt;sup&gt;2&lt;/sup&gt;</td>
<td>38.1&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>44.4</td>
<td>4.9</td>
<td>71.7</td>
</tr>
<tr>
<td>3</td>
<td>101.8</td>
<td>20.1</td>
<td>115.2</td>
</tr>
<tr>
<td>4</td>
<td>73.5</td>
<td>8.8</td>
<td>147.6</td>
</tr>
<tr>
<td>5</td>
<td>68.8</td>
<td>10.4</td>
<td>113.7</td>
</tr>
<tr>
<td>6</td>
<td>207.6</td>
<td>44.2</td>
<td>319.6</td>
</tr>
<tr>
<td>7</td>
<td>349.6</td>
<td>66.3</td>
<td>207.9</td>
</tr>
<tr>
<td>8</td>
<td>111.0</td>
<td>6.5</td>
<td>76.0</td>
</tr>
<tr>
<td>9</td>
<td>44.7</td>
<td>15.5</td>
<td>72.3</td>
</tr>
<tr>
<td>10</td>
<td>251.9</td>
<td>49.1</td>
<td>357.8</td>
</tr>
<tr>
<td>11</td>
<td>166.6</td>
<td>18.2</td>
<td>130.5</td>
</tr>
<tr>
<td>12</td>
<td>68.9</td>
<td>35.7</td>
<td>90.9</td>
</tr>
</tbody>
</table>

Mean ± SEM  
131.5 ± 27.3  24.0 ± 5.8  145.1 ± 29.1

<sup>1</sup>Calculated as nmol·h/µmol β-carotene reference dose from the AUC corrected for trace amount of β-carotene equivalents (all values in this column).
Calculated as nmol·h/µmol β-carotene equivalents \((trans\ \beta\text{-carotene}\ plus\ 50\%\ of\ \textit{cis}\ \beta\text{-carotene}

plus \textit{trans}\ \alpha\text{-carotene},\ and\ 25\%\ of\ \textit{cis}\ \alpha\text{-carotene}\ consumed)\) (all values in this column).

Calculated as nmol·h/µmol vitamin A reference dose (all values in this column).
### TABLE 3

Calculated conversion of the provitamin A carotenoids consumed in the biofortified banana fruit to vitamin A

<table>
<thead>
<tr>
<th>Participant</th>
<th>βC reference dose</th>
<th>Biofortified</th>
<th>Conversion factor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Retinol formed[^1^]</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><code>nmol</code></td>
<td><code>by weight</code></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>2.29</td>
<td>0.18</td>
<td>0.80[^2^]</td>
</tr>
<tr>
<td>2</td>
<td>0.61</td>
<td>0.05</td>
<td>3.00</td>
</tr>
<tr>
<td>3</td>
<td>0.87</td>
<td>0.14</td>
<td>2.10</td>
</tr>
<tr>
<td>4</td>
<td>0.49</td>
<td>0.05</td>
<td>3.73</td>
</tr>
<tr>
<td>5</td>
<td>0.60</td>
<td>0.07</td>
<td>3.07</td>
</tr>
<tr>
<td>6</td>
<td>0.64</td>
<td>0.11</td>
<td>2.86</td>
</tr>
<tr>
<td>7</td>
<td>1.65</td>
<td>0.25</td>
<td>1.10</td>
</tr>
<tr>
<td>8</td>
<td>1.43</td>
<td>0.07</td>
<td>1.27</td>
</tr>
<tr>
<td>9</td>
<td>0.61</td>
<td>0.17</td>
<td>3.00</td>
</tr>
<tr>
<td>10</td>
<td>0.69</td>
<td>0.11</td>
<td>2.64</td>
</tr>
<tr>
<td>11</td>
<td>1.25</td>
<td>0.11</td>
<td>1.46</td>
</tr>
<tr>
<td>12</td>
<td>0.74</td>
<td>0.31</td>
<td>2.45</td>
</tr>
<tr>
<td>Mean ± SEM</td>
<td>0.99 ± 0.16</td>
<td>0.13 ± 0.02</td>
<td>2.29 ± 0.27[^3^]</td>
</tr>
</tbody>
</table>

[^1^]: Retinol formed
[^2^]: Conversion factor
[^3^]: Mean ± SEM
1Calculated as nmol retinol formed/nmol β-carotene equivalents (trans- β-carotene plus 50% of cis-β-carotene plus trans α-carotene, and 25% of cis-α-carotene ingested).

2Calculations were applied to correct for the trace amount of β-carotene equivalents in the wild-type banana (all values this column).

3Significantly different from provitamin A-biofortified banana when compared by using a paired t test, $P < 0.001$
TABLE 4
Inter-assay precision of the target analytes in the aliquots of the pooled quality control plasma triacylglycerol-rich lipoprotein fraction

<table>
<thead>
<tr>
<th></th>
<th>α-Retinyl palmitate/ d8-α-retinyl palmitate</th>
<th>Retinyl palmitate/ 13C_{10}-retinyl palmitate</th>
<th>α-Carotene/ 13C_{10}-β-Carotene</th>
<th>β-Carotene/ 13C_{10}-β-Carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>pmol on column</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Means ± SDs(^1)</td>
<td>0.165 ± 0.017</td>
<td>1.186 ± 0.059</td>
<td>1.439 ± 0.198</td>
<td>1.881 ± 0.138</td>
</tr>
<tr>
<td>Relative SD (%)</td>
<td>10.1</td>
<td>4.9</td>
<td>13.8</td>
<td>7.4</td>
</tr>
</tbody>
</table>

\(^1\)Means and SDs are quality control values from 36 separate analytical runs. \( n = 36 \).
TABLE 5

Maximum content ($C_{\text{max}}$), time of maximal content ($T_{\text{max}}$), and area under the curve values (AUC) for $\alpha$-retinyl palmitate and retinyl palmitate

<table>
<thead>
<tr>
<th>Participant</th>
<th>$C_{\text{max}}$</th>
<th>$T_{\text{max}}$</th>
<th>(AUC)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$\alpha$-Retinyl</td>
<td>Retinyl</td>
<td>$\alpha$-Retinyl</td>
</tr>
<tr>
<td>1</td>
<td>6.5</td>
<td>12.8</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>3.0</td>
<td>3.7</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>4.5</td>
<td>11.8</td>
<td>3.5</td>
</tr>
<tr>
<td>4</td>
<td>12.9</td>
<td>22.4</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>9.7</td>
<td>13.8</td>
<td>5</td>
</tr>
<tr>
<td>6</td>
<td>16.6</td>
<td>29.1</td>
<td>5</td>
</tr>
<tr>
<td>7</td>
<td>36.4</td>
<td>62.7</td>
<td>5</td>
</tr>
<tr>
<td>8</td>
<td>4.4</td>
<td>5.1</td>
<td>5</td>
</tr>
<tr>
<td>9</td>
<td>4.7</td>
<td>11.9</td>
<td>3.5</td>
</tr>
<tr>
<td>10</td>
<td>14.3</td>
<td>18.9</td>
<td>5</td>
</tr>
<tr>
<td>11</td>
<td>4.6</td>
<td>10.2</td>
<td>3.5</td>
</tr>
<tr>
<td>12</td>
<td>7.7</td>
<td>13.8</td>
<td>5</td>
</tr>
</tbody>
</table>
Figures

Figure 1. CONSORT flow diagram of participant recruitment procedures and reasons for exclusions. CONSORT, Consolidated Standards of Reporting Trials.
Figure 2. HPLC-PDA carotenoid chromatographic profile of the biofortified cooked banana fruit detected at 286 nm, 348 nm, and 453 nm. Peaks: 1, lutein; 2, phytoene; 3, phytofluene; 4, 13-cis-α-carotene; 5, 13′-cis-α-carotene; 6, 15-cis-β-carotene; 7, 13-cis-β-carotene; 8, all-trans-α-carotene; 9, 9-cis-α-carotene; 10, all-trans-β-carotene; 11, 9-cis-β-carotene; 12, γ-carotene (internal standard).
Figure 3. Overlay of HPLC-QTOF extracted ion chromatograms of a 3.5-h postprandial triacylglycerol-rich lipoprotein fraction isolated after a participant consumed a biofortified banana test meal. Extracted ion chromatograms at 1) m/z 269.2 for retinyl esters and their α-analogues, 2) m/z 277.2 for the d8-α-retinyl palmitate internal standard, 3) m/z 279.2 for the 13C10-retinyl palmitate internal standard, 4) m/z 536.4 + 537.4 for ε-, α- and β-carotene, 5) m/z 546.4 for 13C10-β-carotene internal standard. Peaks: 1, α-Retinyl linoleic (α-RLO); 2, Retinyl linoleic (RLO); 3, α-Retinyl oleate (α-RO); 4, Retinyl oleate (RO); 5, α-Retinyl palmitate (α-RP); 6, Retinyl palmitate (RP); 7, d8-α-Retinyl palmitate; 8, 13C10-Retinyl palmitate; 9, ε-Carotene; 10, α-Carotene; 11, α-Retinyl stearate (α-RS); 12, Retinyl stearate (RS); 13, 13C10-β-Carotene; 14, β-Carotene.
Figure 4. Mean baseline-corrected plasma triacylglycerol-rich lipoprotein concentrations of α-retinyl palmitate and retinyl palmitate over the course of 9 h after consumption of biofortified cooked banana fruit. Shown are concentrations of analytes reported in means ± SEMs, n = 12.
Appendix A. Foods to avoid

CAROTENOID-RICH FOODS TO AVOID

Study Days 1-3

Fruits:
Apricots
Cantaloupe
Mango
Papaya

Vegetables:
Beet greens
Broccoli
Brussels sprouts
Carrots
Collard greens
Corn
Endive
Kale
Lettuce
Mustard greens
Peas
Peppers, red
Pumpkin
Spinach
Sweet potatoes, yams
Swiss chard
Tomatoes, tomato sauce, tomato juice
Turnip greens
Vegetable soup
Winter squash

Allowed foods include: bananas, blueberries, celery, cherries, cucumbers, grapefruit, grapes, honeydew melon, mushrooms, olives, onions, plums, raspberries, strawberries, white potatoes
VITAMIN A-RICH FOODS TO AVOID
Study Days 1-3

Animal products:

Eggs
Butter
Cheese (limit: 1 ounce per day)
Herring
Liver and other organ meats
Mackerel
Milk (limit: 4 ounces/day)
Milk products (including ice cream)
Salmon
Other products:
Margarine
Foods fortified with vitamin A, including some breakfast cereals, oatmeal, and snack bars
Appendix B. LC-MS operating conditions and mobile phase systems

TABLE 6

LC-MS operating conditions for the provitamin A carotenoid analyses of the triacylglycerol-rich lipoprotein fractions

<table>
<thead>
<tr>
<th>Operating Parameter</th>
<th>Operating Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Column</td>
<td>YMC C30 Carotenoid Column</td>
</tr>
<tr>
<td>Column length and particle size</td>
<td>250 x 4.6 mm, 3 µm</td>
</tr>
<tr>
<td>Column temperature</td>
<td>40°C</td>
</tr>
<tr>
<td>Mobile phase flow rate, mL/min</td>
<td>1.2</td>
</tr>
<tr>
<td>Injection volume, µL</td>
<td>50</td>
</tr>
<tr>
<td>Mass spectrometry detection</td>
<td>ESI (+)</td>
</tr>
<tr>
<td>Gas temperature, °C</td>
<td>350</td>
</tr>
<tr>
<td>Drying gas, L/min</td>
<td>12</td>
</tr>
<tr>
<td>Nebulizing pressure, psi</td>
<td>45</td>
</tr>
<tr>
<td>Capillary voltage, V</td>
<td>3500</td>
</tr>
<tr>
<td>Nozzle voltage, V</td>
<td>250</td>
</tr>
</tbody>
</table>
TABLE 7

HPLC-QTOF gradient designs A) pre-run and acquisition gradient, B) wash system, for the analyses of provitamin A carotenoids in the triacylglycerol-rich lipoprotein.

A: Pre-run and acquisition gradient

<table>
<thead>
<tr>
<th>Mobile Phase Components</th>
<th>A = MeOH: ACN: H2O 65:25:10 (with 5 mM ammonium formate)</th>
<th>B = IPA: ACN: MTBE 50:25:25 (with 5 mM ammonium formate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-run Mobile Phase Gradient</td>
<td>Time</td>
<td>% B</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0</td>
</tr>
<tr>
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B: Wash system

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Appendix C. Vitamin A equivalence calculations

Vitamin A equivalence calculations

The following equation was used to correct for the vitamin A contribution of the provitamin A carotenoids in the wild-type fruit to the total vitamin A absorbed after consumption of the wild-type banana fruit portion with the added β-carotene or vitamin A reference dose. This calculation is based on the basis that the provitamin A carotenoids in the wild-type banana fruit with vitamin A reference dose provides the same vitamin A equivalence as the provitamin A carotenoids in the biofortified banana fruit. The total vitamin A contribution from consumption of the wild-type banana with vitamin A reference dose can then be represented as follows:

\[
\text{Total vitamin A (ng) in the wild-type banana with vitamin A reference dose} = \text{vitamin A reference dose (ng)} + \frac{\beta\text{-carotene equivalents in wild-type banana (ng)}}{\text{Vitamin A equivalence of biofortified banana}} \tag{1}
\]

Therefore, the corrected equation is as follows:

The corrected vitamin A equivalence (by wt) =

\[
\frac{\text{Retinyl palmitate AUC after ingestion of the wild-type banana with vitamin A reference dose}}{\text{Retinyl palmitate AUC after ingestion of the biofortified banana}} \times \frac{\beta\text{-carotene equivalents in biofortified cooked banana fruit (ng)}}{\text{vitamin A reference dose (ng)} + \frac{\beta\text{-carotene equivalents in wild-type banana (ng)}}{\text{Vitamin A equivalence of biofortified banana (ng)}}} \tag{2}
\]

The simplified form is as followed:

The trace amount corrected vitamin A equivalence (by wt) =

\[
\frac{\text{Retinyl palmitate AUC after ingestion of the wild-type banana with vitamin A reference dose}}{\text{Retinyl palmitate AUC after ingestion of the biofortified banana}} \times \frac{\beta\text{-carotene equivalents in biofortified banana (ng)}}{\text{Vitamin A equivalence of biofortified banana (ng)}} - \frac{\beta\text{-carotene equivalents in wild-type banana (ng)}}{\text{Vitamin A equivalence of biofortified banana (ng)}} \tag{3}
\]

Because the presence of a trace amount of provitamin A in the wild-type banana contributes to the retinyl palmitate formed as measured by the AUC, we also account for the retinyl palmitate
AUC formed by the contribution of provitamin A alone in the wild-type banana with β-carotene reference dose. With the same assumption made, we utilized vitamin A equivalence of biofortified banana to calculate the retinyl palmitate AUC formed from the contribution of provitamin A alone after the ingestion of wild-type banana with β-carotene reference dose.

The retinyl palmitate AUC formed from the provitamin A alone in the wild-type banana =

\[
\frac{\text{Retinyl palmitate AUC from vitamin A reference dose}}{\text{Vitamin A equivalence of biofortified banana}} \times \frac{\beta\text{-carotene equivalents in wild-type banana}}{\text{Vitamin A reference dose (ng)} + \frac{\beta\text{-carotene equivalents in wild-type banana (ng)}}{\text{Vitamin A equivalence of biofortified banana (ng)}}}
\]

(4)

Actual retinyl palmitate AUC formed from β-carotene reference dose after ingesting the wild-type banana with β-carotene reference dose =

\[
\text{Retinyl palmitate AUC after ingestion of the wild-type banana with β-carotene reference dose} - \text{Retinyl palmitate AUC formed from the provitamin A alone in the wild-type banana}
\]

(5)

Therefore:

\[
\frac{\text{Vitamin A equivalence of provitamin A in wild-type banana with β-carotene reference dose}}{\text{Retinyl palmitate AUC after ingestion of the wild-type banana with vitamin A reference dose}} = \frac{\text{Actual retinyl palmitate AUC formed from β-carotene reference dose after ingesting the wild-type banana with β-carotene reference dose}}{\beta\text{-Carotene reference dose (ng)} \times \frac{\beta\text{-carotene equivalents in wild-type banana (ng)}}{\text{Vitamin A equivalence of biofortified banana (ng)}}}
\]

\[
\frac{\text{Vitamin A reference dose (ng)} + \frac{\beta\text{-carotene equivalents in wild-type banana (ng)}}{\text{Vitamin A equivalence of biofortified banana (ng)}}}{\text{Vitamin A equivalence of biofortified banana (ng)}}
\]
CHAPTER 5. GENERAL CONCLUSION

The studies presented in this dissertation have shown that provitamin A carotenoid-biofortified bananas have the potential to be an effective vehicle to deliver provitamin A carotenoids as a dietary source of vitamin A to vitamin A-deficient populations. The biotechnology used to accumulate provitamin A in the low-provitamin A Cavendish banana fruit as a proof of concept will be transferred to plantain cultivars, which are a staple food widely consumed by vitamin A-deficient Ugandan children as a popular weaning food “Matooke”. Because provitamin A carotenoid bioavailability is greatly impacted by the plant matrix, an accurate bioconversion factor in humans is needed to determine if the provitamin A carotenoid biofortified banana fruit has achieved an optimal target β-carotene equivalent content that is sufficient to improve the micronutrient status. Our study provides a finding of significance to the plant breeders, which is that the biofortified banana fruit has a vitamin equivalence similar to that of the provitamin A carotenoids in carotenoid-rich vegetables, such as carrots and spinach (1).

Provitamin A-biofortified bananas contain predominantly α-carotene, followed by β-carotene. There is limited knowledge regarding factors affecting the metabolism of α-carotene within the body and the vitamin A activity of α-carotene in α-carotene-containing foods. However, the limited biological activity of the cleavage product of α-carotene (i.e., α-retinal) is well-established in animal models (2,3). Its postprandial appearance has been thought to contribute to error in analysis in the determination of the vitamin A equivalence of provitamin A carotenoid-enhanced foods. The cleavage of α-carotene produces α-retinal, which can be converted to α-retinol. α-Retinol does not bind to retinol-binding protein for cellular uptake but circulates as α-retinyl esters (4). Due to the ε-ionone ring, α-retinol lacks vitamin A activity.
We successfully developed an ultra-highly selective and sensitive quantitative approach using HRMS technology to capture and quantify the low abundance of α-retinyl esters and retinyl esters after the ingestion of the α-carotene-containing biofortified bananas. We have shown that HPLC-HRMS-QTOF operated in ESI positive mode can be used as a quantitative platform to measure the postprandial appearance of provitamin A carotenoids and their respective retinyl ester bioconversion products. With the use of stable-isotope-labeled internal standards, we have demonstrated that HRMS, specifically QTOF, is a state-of-the-art quantitative tool for bioanalysis. The accurate mass measurement offered by HRMS allows the identification of isobaric compounds if they coelute with the target analytes within the complex matrix. Because the conventional mobile phase for vitamin A analysis is not suitable for ESI ionization on the LC-HRMS, we developed a novel alternative mobile phase system. The new system showed significant improvement in the sensitivity of detection of the analytes of interest in our study compared with the conventional C30 Carotenoid Column mobile phase system. We investigated three other buffer additive options and discovered that an ammonium formate-containing buffer increases sensitivity when using the ESI-compatible mobile phase system compared to using formic acid alone as the buffer. HRMS technology achieved the sensitivity to quantify the postprandial appearance of provitamin A carotenoids after the consumption of physiological dosage of provitamin A-enriched foods.

We employed the optimized HPLC-ESI(+)−HRMS-QTOF analytical method we developed to quantify the postprandial absorption of provitamin A carotenoids and their respective retinyl ester bioconversion products in the triacylglycerol-rich-lipoprotein fractions of 12 healthy women after the ingestion of 1) provitamin A carotenoid biofortified cooked banana fruit, 2) the wild-type cooked banana fruit with vitamin A reference dose, and 3) the wild-type cooked banana fruit with β-carotene reference dose. By capturing the
postprandial appearance of these analytes in the TRL fractions, we successfully determined the vitamin A equivalence of biofortified bananas to be 18.5 µg to 1 µg of retinol. In addition, we validated our experimental approach by showing an expected equivalency of 2 µg to 1 µg of retinol for the β-carotene reference dose.

The findings of this dissertation can be summarized as follows:

1. ESI(+) ion source-specific mass spectrometric parameters, solvent composition, and buffer additive are important to the accuracy, precision, and sensitivity of the analysis.

2. The ESI linear dynamic range can be extended by using stable-isotopic labeled internal standards. The limited dynamic range of ESI can be overcome by the use of appropriate stable-isotopic labeled internal standards.

3. Stable isotope-labeled internal standards correct for background interference and are therefore advantageous for plasma vitamin A and carotenoid quantitation.

4. The optimized HPLC-ESI(+)-HRMS-QTOF method has ultra-high sensitivity to simultaneously identify and quantify the postprandial appearance of provitamin A carotenoids and their respective retinyl ester bioconversion products in plasma TRL fractions.

5. An average of 18.5 µg of β-carotene equivalents in the cooked biofortified bananas is required to provide 1 µg of retinol. This bioconversion factor reflects the complex plant matrix where the provitamin A carotenoids are embedded.

6. The 200-g provitamin A carotenoid biofortified banana fruit portion used in this study can provide 15% of the Estimated Average Requirement (EAR) of an adult woman.

**Strengths and limitations**

We provided a cost-effective human study utilizing a cross-over study design in a controlled environment to examine the bioefficacy of a biofortified crop in healthy women, where the measurement only shows the intestinal conversion of provitamin A to vitamin A.
The measurement of the postprandial retinyl ester response after the ingestion of provitamin A biofortified crops can act as a screening tool before conducting a large expensive bioefficacy field trial. Understanding the plant matrix effect on the bioavailability of provitamin A carotenoid, and other micronutrients is an essential step to estimate the target level to which the nutrients can be bred into crops in order to improve vitamin A status.

Although the amount of β-carotene equivalents consumed in the biofortified bananas (1415 µg/200 g βCE) was 2.6-times higher than the β-carotene reference dose (536.8 µg/200 g), each participant did not produce more retinyl palmitate after the ingestion of provitamin A biofortified bananas. Several speculations may be associated with the lower bioavailability in the banana fruit, which includes the plant matrix and/or the genetic variation of the host. We validated the study experimental design with the β-carotene reference dose added to the wild-type bananas. The vitamin A equivalence of β-carotene in oil determined in the present study was in agreement with the National Academies of Medicine, i.e., 2 µg of β-carotene in oil is required to provide 1 µg of retinol.

Secondly, we highlighted the use of QTOF-HRMS technology as a quantitative tool for bioanalysis when appropriate stable-isotope-internal standards are used to compensate for analytical variability. It has been a paradigm to believe QTOF is a qualitative analytical tool for structural elucidation in untargeted metabolomic studies. However, we have demonstrated that QTOF is not only the gold standard for untargeted analysis but can be used as a quantitative tool for targeted analysis. Thirdly, we discovered that the conventional mobile phase used for vitamin A LC-analysis is not an ESI-compatible system, and therefore, an alternative mobile phase system was developed. This observation highlighted the importance of method development for each LC-MS analysis; thus there is no one method that fits all in LC-MS analysis.
**Future research**

Biofortified staple crops are a promising tool to improve human health and nutrition. These crops, when consumed cooked daily, can provide adequate micronutrients to meet the daily requirement for normal biological functions. An animal study reported that the bioavailability of provitamin A in the banana fruit in the cooked fruit was higher than the raw fruit when fruits were fed to Mongolian Gerbil (5). Existing studies have shown that biofortified foods widely adopted by target populations produce improvement in vitamin A status (6). Hence, this could also include biofortified bananas, especially when the optimal breeding target provitamin A content is achieved. There is limited information in the research literature that explains the large inter-individual variability of \( \alpha \)-carotene conversion efficiency and the association of genetic variants with \( \alpha \)-carotene metabolism. Assessing the SNPs that are related to the metabolism of \( \alpha \)- and \( \beta \)-carotene metabolism can further define the mechanism of \( \alpha \)-carotene uptake and conversion efficiency. Moreover, the use of ultra-high sensitivity instrumentation to measure the postprandial responses of provitamin A carotenoids and their bioconversion product is important. Other possible work may include the investigation of an alternative analytical column that allows a shorter analysis time. A shorter C30 Carotenoid Column with a smaller internal diameter and pore size would allow the utilization of ultra-high-pressure liquid chromatography (UHPLC).

**References**


