# Effect of processing and meal composition on bioaccessibility and bioavailability of dietary carotenoids following simulated digestion.


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# Abstract

Dietary approaches aimed at combating vitamin A deficiency (VAD) through consumption of provitamin A carotenoids, in the form of biofortified foods, are becoming increasingly popular because of their assumed efficacy in addressing this challenge. This is on the premise that, regular consumption of biofortified staple foods, would contribute significantly to the vitamin A body stores of vitamin A deficient individuals. However, recent studies have demonstrated that content alone, cannot be relied upon completely as processing, food preparation and meal composition have been reported to have a greater effect on nutrient bioaccessibility and eventual delivery for intestinal absorption. In this study, the role of food processing and preparation (baking, boiling and composite meal preparation) methods in modulating the bioaccessibility and potential bioavailability of dietary carotenoids following simulated *in vitro* gastro-intestinal digestion was investigated. Test foods prepared from biofortified orange-fleshed sweet potato (OFSP) and maize foods, with varying degrees of caloric content, were subjected to both static and semi-dynamic digestions models for bioaccessibility and efficiency of micellarisation assessement. The resulting chyme was then exposed to differentiated Caco-2 cells and murine intestinal tissue to assess the potential bioavailability of dietary carotenoids using HPLC and LCS-MS analytical procedures. From the test foods analysed, processing led to a significant reduction in the contents of all the dietary carotenoids ($P < 0.05$) except for the β-carotene isomers, 13-*cis*-β-carotene and 9-*cis*-β-carotene. This is possibly due to trans-*cis*-isomerisation and potential degradation of the carotenoids. When different digestion models were compared, the efficiency of micellarisation, and therefore bioaccessibility for all-*trans*-β-carotene was significantly greater among the provitamin A carotenoids using the semi-dynamic (66.0 ± 2.0%) compared to the static method (58.0 ± 5.0%) ($P < 0.05$). Similarly, the micellarisation efficiency was greatest for lutein when assessed with the semi-dynamic method (75.3 ± 6.2%) compared to the static method (60.2 ± 7.1%) ($P < 0.05$). On the relationship between caloric density and bioaccessibility, addition of 5% vegetable oil to the OFSP (LIPID+OFSP) led to a two-fold increase in the bioaccessibility of both carotenoids (25 to 49%) for all-*trans*-β-carotene and 30 to 57% for β-cryptoxanthin. However, further addition of 5% protein (LIPID+OFSP+PROTEIN) led not only to an increase in the caloric density but also delayed delivery to the intestinal phase and thus reduction in the bioaccessibility in both carotenoids. Results of this study thus demonstrate how modification of food composition, through addition of moderate lipid and protein, in biofortified food material can improve the bioaccessibility and subsequent bioavailability of dietary carotenoids therefore reduce vitamin

A deficiency in target populations. To validate these findings, carefully controlled human studies are needed using isotopic tracer methods to identify how meal composition modulates stability, digestion and bioavailability of dietary carotenoids in the body. This data can then be used by policy implementers to design and promote locally accepted meals, with the right energy composition, aimed at reducing the prevalence of vitamin A deficiency in afflicted populations.

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# List of Abbreviations

| | |
|---|---|
| ALP | Alkaline Phosphatase |
| ANOVA | Analysis of Variance |
| AUC | Area Under The Curve |
| BCO1 | Beta Carotene 15,15’ Oxygenase-1 |
| BCO2 | Beta Carotene 9’,10’ Oxygenase-2 |
| BHT | Butylated Hydroxytoluene |
| BNSQ | Butternut Squash |
| CD36 | Cluster of Differentiation 36 |
| CIAT | International Centre for Tropical Agriculture |
| CV | Coefficient of Variation |
| DAD | Diode Array Detector |
| DMEM | Dulbecco’s Modified Eagle’s Medium |
| DMSO | Dimethyl Sulfoxide |
| DNSA | 3,5-Dinitrosalicyclic Acid |
| DPBS | Dulbecco’s Phosphate Buffer Saline |
| DW | Dry Weight |
| ESI | Electrospray Ionisation |
| FBS | Fetal Bovine Serum |
| GE | Gastric Emptying |
| GI | Gastro-intestinal |
| HCl | Hydrochloric Acid |
| HPLC | High Performance Liquid Chromatography |
| ICRISAT | International Crops Research Institute for Semi-Arid Tropics |
| IOM | Institute of Medicine |
| IU | International Units |
| KOH | Potassium Hydroxide |
| LCMS | Liquid Chromatograph Mass Spectrophotometry |
| LOD | Limit of Detection |
| LOQ | Limit of Quantification |
| LRAT | Lecithin Retinol Acyltransferase |
| LY | Lucifer Yellow |

| | |
|---|---|
| MTBE | Methyl Tert-Butyl Ether |
| MTT | 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide |
| NPC1L1 | Niemann-Pick C1-Like1 |
| PD | Potential Difference |
| P-NP | Para-Nitrophenol |
| P-NPP | Para-Nitrophenyl Phosphate |
| Pro-VITA | Provitamin A |
| PTFE | Polytetrafluoroethylene |
| RAE | Retinol Activity Equivalent |
| RP | Reverse Phase |
| SD | Standard Deviation |
| SIM | Selected Ion Mode |
| SRBI | Scavenger Receptor Class B Type 1 |
| SRM | Selected Reaction Monitoring |
| SNP | Single Nucleotide Polymorphism |
| TAME | *p*-Toluene-Sulfonyl-$_L$-Arginine Methyl Ester |
| TCA | Trichloroacetic Acid |
| TEER | Trans-epithelial Electrical Resistance |
| TRL | Triacyl-glycerol Rich Lipoprotein |
| TIC | Total Ion Chromatography |
| VAD | Vitamin A Deficiency |
| WHO | World Health Organisation |
| ZARI | Zambia Agriculture Research Institute |

# CHAPTER 1

# Introduction

## 1.1 Background

Vitamin A is an essential vitamin that is involved in several physiological processes such as general growth, maintenance of visual function, cell differentiation, immune function and embryonic development (Miller et al., 2020; Tang and Russell, 2004). VAD is one of the most prevalent micronutrient deficiency disorders that continues to impose significant health challenges worldwide (Priyadarshani, 2017) and is caused by inadequate dietary intake of vitamin A. As the human body cannot synthesise its own vitamin A, adequate amounts have to be obtained through dietary intake of foods from either animal sources or through the consumption of foods fortified with vitamin A (Reboul and Borel, 2011). According to the World Health Organisation (WHO), the problem of VAD is particularly prevalent in low income countries and based on the currently accepted cut-off for low serum retinol concentrations (<0.7 μmol/L), affects an estimated 190 million preschool-aged children and 19 million pregnant women annually (WHO, 2009). Of these, nearly 100 million live in south Asia and sub-Saharan Africa were severity of the disease has led to stunted growth, weak immunity, xerophthalmia and high mortality rates (Humphrey et al., 1996; Mason et al., 2001; van Jaarsveld et al., 2005; Harrison, 2012; Tanumihardjo et al., 2016).

Attempts to combat VAD are currently being addressed by most countries afflicted by the disease through strategies that include supplementation of high dose vitamin A in children under the age of 5 and post-partum mothers as the main strategy (Bastos Maia et al., 2019). Food fortification using preformed vitamin A in foods like sugar, cereals and cooking oil as delivery vehicles to reach vulnerable populations, promotion of dietary diversity and more recently, biofortification through nutrient enrichment of staple foods are the other strategies that have been considered (Rodriguez-Amaya, 2015).

## 1.2 Strategies to combat vitamin A deficiency

While no single strategy can be advocated for as the best in combating micronutrient deficiency, a combination of two or more of the following methods have been demonstrated to produce good results (Tanumihardjo et al., 2016);

### 1.2.1 Vitamin A capsule supplementation

Vitamin A capsule supplementation, using an oil-based preparation of retinyl palmitate, is the recommended and cost-effective strategy in select groups afflicted with acute VAD (Rolls, 2009; Tanumihardjo et al., 2016). This is because it is inexpensive, quick and an effective way to improve vitamin A status, reduce child morbidity and mortality in the long term (WHO, 2009). Under this program, children aged 6 months to 5 years receive high doses of vitamin A capsules (100,000 international units (IU) for children 6 to 11 months and 200,000 IU for children 1-5 years) at 6 months intervals (Rodriguez-Amaya, 2015). These oil-based vitamin A supplements are delivered using gelatin capsules, as a single dispenser or graduated spoon (WHO, 2010). The effectiveness of this intervention was demonstrated in population based-intervention studies conducted in southeast Asia, south Asia, and Africa suggesting that high-dose vitamin A supplementation with 200,000 IU as retinyl palmitate every 6 months, could reduce mortality among children aged 6-59 months by 6-54% (Tanumihardjo et al., 2016). Despite the programs' demonstrated ability to reduce childhood mortality, operational challenges related to its implementation, particularly in hard to reach places (Rodriguez-Amaya, 2015), that also happen to be the most affected with the disease, the risk of over-supplementation such as overlapping supplementation efforts and/or with combinations with food fortification programs remain areas of concern (Chea et al., 2020).

### 1.2.2 Food fortification with vitamin A

In both developed and developing countries, fortification of foods as a strategy in mitigating micronutrient related challenges, has successfully been used. According to the WHO guidelines on food fortification with micronutrients, fortification of food with preformed vitamin A is a viable option that can be 2-4 times more cost-effective in providing vitamin A than either capsule distribution with preformed retinyl palmitate or dietary diversification efforts (WHO, 2006). While this method has the advantage of being socially acceptable and requires little to no changes in dietary habits, concerns of indiscriminate fortification of foods that can potentially result in over fortification or under fortification in the food supply vehicle and create nutrient imbalances in the diets of targeted populations have been noted (Dwyer et al., 2014).

### 1.2.3 Dietary diversification

Despite difficulties in evaluating their efficacies, diversification of dietary intake is a strategy that has been identified and used to combat micronutrient deficiency disorders. The approach is culturally acceptable and presents the advantage that bioactive

compounds present in the food material would exert their nutritional and health related benefits in unison with other compounds within the food matrix. A study by Greiner (2013) found that dietary diversity, as a food-based approach, was able to promote self-sufficiency and food security as opposed to perpetual dependency on donor-driven programs that cannot be sustained in the long term.

### 1.2.4 Biofortification

Nutrient enrichment of staple crops using the best traditional practices, and in some cases modern biotechnology to achieve provitamin A concentrations that can have measurable effects on human health and nutritional status is a process known as biofortification (Kimura et al., 2007). As a recent approach, the process is intended to complement existing strategies meant to reduce micronutrient deficiency disorders in populations afflicted with VAD. Since biofortification happens in staple crops relied upon by the majority of populations, especially in poorer countries, it is assumed that daily consumption of the biofortified staple food would provide the necessary micronutrient on a regular basis and therefore meet the body's target nutrient levels in the long run (Rodriguez-Amaya, 2015). The same authors further estimate that biofortified foods like orange-fleshed sweet potato (OFSP) are expected to provide 50-100% of the daily vitamin A needs when consumed on a regular basis.

Some challenges regarding effective implementation of the given strategies in mitigating VAD have been observed. These include possible cases of hypervitaminosis A arising from multiple fortification programs and sustainability issues considering that most programs are donor driven. With this background, only dietary diversity and biofortification approaches appear cost-effective and socially acceptable in afflicted communities. This is particularly true as over 80% of vitamin A needs are met through dietary intake of foods rich in provitamin A carotenoids (Blomme et al., 2020). However, most questions on process and product-related factors and how these influence the conversion of carotenoids to vitamin A remain unanswered. Indeed, knowledge about the food processing and preparation methods and how these modulate the release and absorption of provitamin A carotenoids during GI digestion needs urgent investigations in order to improve their bioavailability and potentially vitamin A status in individuals. This is only possible if the general chemistry, occurrence and potential benefits of these compounds to human health are clearly understood.

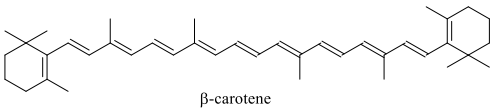
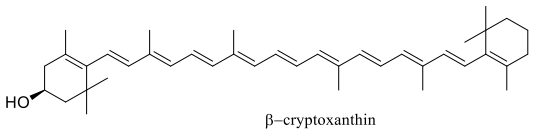
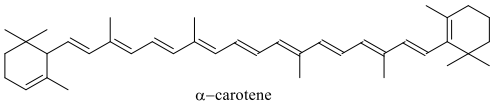
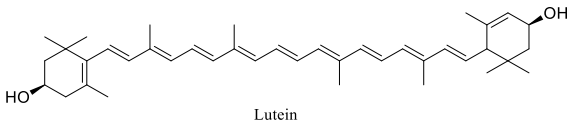
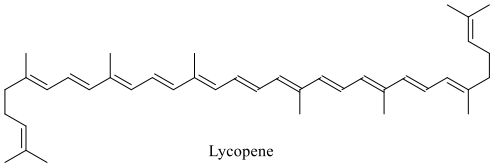
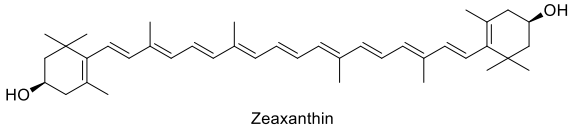
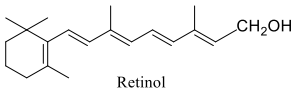
## 1.3 Carotenoids and their chemistry

Carotenoids are C40 isoprenoids compounds that are widely distributed in nature and synthesised by plants and different types of microorganisms such as bacteria, fungi and algae. To date, more than 700 carotenoids have been isolated from natural sources out of which ~60 are found in the human diet and ~20 can be detected in human blood and tissue (Harrison and Kopec, 2018; Trono, 2019). These pigments are responsible for the yellow, orange and red colour in many fruits, vegetables, plumage in birds and other marine animals (Kopec et al., 2012; Harrison and Kopec, 2018). Carotenoids are highly unsaturated compounds composed either entirely of carbon and hydrogen (carotenes) or carbon, hydrogen and oxygen (xanthophylls), a classification that determines their natural functions and properties (Kopec et al., 2012; Saini et al., 2015). The conjugated polyene chromophore presented in the carotenoid molecule determines its light absorption, light harvesting properties, provision of both compound colour and photo-protection properties (Jiao et al., 2019; Saini et al., 2015).

### 1.3.1 Structure of carotenoids

On the basis of their structure, major carotenoids can be divided into two structurally different families divided into the following subclasses: carotenes, composed of only unsaturated hydrocarbons (e.g., α-, β-carotene and lycopene), xanthophylls, characterised by oxygenated derivatives (e.g., β-cryptoxanthin, lutein and zeaxanthin) (Courraud et al., 2013; Jiao et al., 2019). The functional groups in xanthophylls are mainly responsible for the degree of polarity, solubility and chemical behaviour (Rodriguez-Amaya, 2015). The structure of carotenoids is as indicated in Figure 1-1.

Table 1-1: Structures of some of the major carotenoids and retinol

| Carotenes | Xanthophylls |
|---|---|
|  β-carotene |  β–cryptoxanthin |
|  α–carotene |  Lutein |
|  Lycopene |  Zeaxanthin |
|  Retinol | |

### 1.3.2 Importance of carotenoids on human health

Apart from their functions in aiding photosynthesis through light absorption and protection against photosensitization in plants (Kopec et al., 2012), carotenoids have been reported as important dietary compounds that have significant health and nutritional benefits in humans (de Oliveira et al., 2020). However, as higher mammals cannot synthesis these compounds *de novo*, intake is therefore through dietary sources particularly from fruits and vegetables (Nagao, 2014; Rodriguez-Amaya, 2015). Several studies have documented the association between the consumption of diets rich in carotenoids and a lower risk of degenerative diseases and certain types of cancers (During and Harrison, 2004; Nagao, 2014; Rodriguez-Amaya, 2015), age-related macular degeneration (Miller et al., 2020), cardiovascular diseases (Desmarchelier and Borel, 2017; Saini and Keum, 2018; Anunciação et al., 2019; Bohn, 2019) their role as vitamin A precursors (Bohn, 2019; Bouis and Saltzman, 2017; Chilungo et al., 2019; De Moura et al., 2015; Taleon et al., 2019). The putative role of carotenoids in preventing and/or counteracting disease has widely been attributed to their antioxidant activities, through quenching of the reactive singlet oxygen and free radical scavenging properties (Kopec et al., 2012). Free radicals or reactive oxygen species have been postulated to cause

damage to biologically important molecules like lipids, proteins and DNA leading to the initiation and progression of chronic diseases (Rodriguez-Amaya, 2015). Other than the indicated health benefits, some carotenoids are also known for their contribution to vitamin A requirement in humans (Moran et al., 2018) only if they meet the following conditions: a carotenoid with an unsubstituted β-ionone ring and a polyene chain containing 11-carbon atoms (Rodriguez-Amaya, 2015). In this regard, the structure of β-carotene entails that it can theoretically generate two molecules of vitamin A (retinol) when centrally cleaved. On the other hand, α-carotene and β-cryptoxanthin each containing one unsubstituted β-ionone ring can only generate one molecule. Therefore, these three carotenoids possess vitamin A activity among the carotenoids found in plant foods and consumed by humans. Conversely, carotenoids devoid of unsubstituted β-ionone ring like lutein, zeaxanthin and lycopene have no provitamin A activity. Their important health benefits to the consumers such as ameliorating age-related macular degeneration have been reported elsewhere (De Moura et al., 2015; Kopec and Failla, 2018).

## 1.4 Bioaccessibility and bioavailability

Before exerting their health and nutritional effects in the human body, carotenoids need to undergo six distinct steps: (1) they have to be released from the food matrix, (2) because they are lipophilic in nature, they need to solubilise in the lipid droplet, (3) incorporated into mixed micelles after stabilisation by the action of biliary salts and pancreatic enzymes in the intestinal lumen, (4) taken up by intestinal mucosal cells (enterocytes), (5) absorbed intact or metabolised into retinol (for provitamin A carotenoids) and (6) secreted into the lymph for use or storage at the site of action (During and Harrison, 2004; Rodriguez-Amaya, 2015; Bohn et al., 2018). Adipose tissues have been reported to be among the relevant sites for carotenoid and retinoid storage and metabolism in mammals (Bonet et al., 2020). The first three steps that involve release and micellarisation of carotenoids are known as bioaccessibility and are defined as the fraction of carotenoids released from the food matrix during intestinal digestion that is available for absorption (Rodriguez-Amaya, 2015). Bioaccessibility studies are screening tools that predict bioavailability and are usually determined using *in vitro* experiments. On the other hand, bioavailability, represented by stages four to six, refer to the fraction of the ingested carotenoids available for physiological utilisation and storage at the site of action (Saini et al., 2015; Bohn, 2019). Bioaccessibility is part of bioavailability and has thus been described as the most critical step that predicts the amount of carotenoids to be taken up by enterocytes for absorption and metabolism. Controlled studies involving human subjects are considered the best models used to determine bioavailability (Saini et al., 2015).

### 1.4.1 Models of determining bioaccessibility

Whereas direct evaluation of carotenoid absorption using human subjects remain ideal models for estimating bioavailability, several limitations related to experimental design and rigorous ethical considerations have been reported in their application (Failla et al., 2008; Saini et al., 2015). Furthermore, the use of human and other animal models when estimating carotenoid content and composition in food does not include the impact of digestion in the gastrointestinal tract. Often, carotenoid content in foods is not necessarily indicative of the quantity to be obtained following digestion and absorption as metabolic products during the process are not taken into consideration. Given these challenges, simple, inexpensive, rapid and reproducible *in vitro* methods have been developed for the screening of carotenoid bioaccessibility (Rodriguez-Amaya, 2015; Saini et al., 2015) whose application is suitable in obtaining information on a sequence of events that modulate different stages of carotenoid bioaccessibility in the human gastrointestinal tract

during digestion. A typical laboratory set-up would employ an *in vitro* digestion model coupled with Caco-2 cells that simulates human oral, gastric, intestinal digestion and absorption. This model has been found to have the advantage of high throughput in its use as many samples can be analysed within a short time (Saini et al., 2015). Currently, there are several *in vitro* digestion protocols that have been used to obtain data able to predict *in vivo* experiments (Garrett et al., 1999; Chitchumroonchokchai et al., 2004; Courraud et al., 2013). These protocols however lack consensus in many aspects such as food preparation methods, presence or absence of the oral phase, type and concentration of enzymes used, pH, duration of both gastric and intestinal digestion and especially on the preparation and isolation of the micellar fraction (Carbonell-Capella et al., 2014; Rodriguez-Amaya, 2015). It is not surprising that such differences have been reported to lead to significant variations in the bioaccessibility results. Recently, Moran et al. (2018) reported that the bioaccessibility of carotenoids is affected by several extrinsic and intrinsic factors that ultimately affect the body's physiologic response. The extrinsic factors are those related to the food preparation and processing methods while intrinsic factors are those related to the fate of carotenoids during absorption such as the transfer of carotenoids from the food matrix to the micelle fractions, uptake of carotenoids by enterocytes, enzymatic cleavage of carotenoids into vitamin A, incorporation into chylomicrons and secretion into the lymph for delivery to the tissues (Aherne et al., 2010; Bohn et al., 2017).

Several *in vitro* models simulating GI digestion have been widely used to study the behaviour of food during the digestion process. Among the common *in vitro* models are static and semi-dynamic models. The static, being simple to use, is based on constant ratios of meal to enzymes, bile acids and other salts at each stage of digestion (Minekus et al., 2014) while the semi-dynamic model, more closely simulates the human gastric physiologic conditions such as the gradual addition of enzymes, gastric juices and emptying is the other (Egger et al., 2019; Mulet-Cabero et al., 2020b; Mulet-Cabero et al., 2017).

### 1.4.2 Static *in vitro* digestion model

Also called biochemical methods, the static *in vitro* digestion protocols are the simplest techniques that simulate the 3 stages of digestion (oral, gastric and intestinal stages). Designed to use standard laboratory equipment without restrictions on experience for its users, the method is known as static as it uses constant ratios of meal to digestive fluids and a constant pH for each step of digestion (Brodkorb et al., 2019). The model has the

advantage that digestive fluids, enzymes used, pH and average duration of digestion is based on available physiological data. Like most *in vitro* models simulating *in vivo* studies, static models have further advantages of being reproducible, can easily be validated and standardised, relatively inexpensive and cost-effective (Verhoeckx et al., 2015). The major disadvantage with static models however, is that they are not dynamic and therefore cannot mimic certain physiological processes such as changes in conditions over time occurring during digestion (Wickham et al., 2009). Because of the limitations associated with *in vitro* static digestion, a more physiologically relevant model known as the *in vitro* semi-dynamic has been proposed.

### 1.4.3 *In vitro* Semi-dynamic digestion model

This model is similar to the static digestion model described in section 1.4.2 in the use of simulated digestive fluids and the mere fact that both simulate upper gastrointestinal digestion. However, the focus for this model is on the role of the gastric phase in modulating nutrient release and absorption from the food matrix through gradual addition of enzymes, acids and emptying of foods from the stomach (Mulet-Cabero et al., 2020b). This method employs a lower final pH of 2 and a variable gastric incubation time owing to factors associated with gastric emptying. The model takes into account particular parameters such as food composition and energy content and how these affect structural changes in food during simulated gastric digestion in estimating the rate and extent of the bioaccessibility of nutrients. Considering the complexity of the food material used in the current study, the semi-dynamic digestion model was used to obtain kinetic data on nutrient bioaccessibility and bioavailability. One major disadvantage with the semi-dynamic digestion model is that it can neither be used to assess uptake and absorption nor measure carotenoid competition at the site of absorption as the case obtains *in vivo*. Since the use of *in vitro* gastrointestinal digestion models are meant to assess process related factors affecting bioavailability, coupling the digestion models with Caco-2 cells or other models to measure absorption maybe crucial in providing fundamental information of carotenoid absorption and intracellular mechanisms governing their uptake and transport across the epithelial tissue (During et al., 2002).

### 1.4.4 Caco-2 cell model

Derived from human carcinoma colon cells, the Caco-2 cells spontaneously differentiate in cell culture to form a monolayer of polarised cells with functional and morphological features typical of absorptive enterocytes with brush border layer as obtained in the small intestines (Verhoeckx et al., 2015; Cilla et al., 2018). Apart from the differentiated cells

being characterised by apical microvilli and intercellular tight junction complexes, the Caco-2 cells express many of the transport and enzyme markers critical in assessing epithelial barrier functionality and transepithelial transport of nutrients. The Caco-2 cells therefore, provide data on preabsorptive events as they relate to the understanding of carotenoid bioavailability. The major advantage with the Caco-2 cells, notwithstanding the absence of the mucin layer, is that they can be used to assess uptake, absorption, transport kinetics and can further be used to measure carotenoid competition at the site of absorption (Verhoeckx et al., 2015).

### 1.4.5 Ussing chamber

The Ussing chamber system is another model that has been used to investigate nutrient transport and bioavailability. Despite being laborious with a low throughput and short term survival in culture (Verhoeckx et al., 2015), the Ussing chamber method has the advantage that it uses real tissue (intestinal segments) containing the physiological and morphological features of the intestines coupled with an interplay of numerous complex processes involved in the transportation and absorption of nutrients. Unlike the Caco-2 cells that are composed of a single layer, the intestinal epithelium used in the Ussing chamber method offers a better representation of the morphological and physiological features of the intestinal wall (Mackie et al., 2020). These include all the relevant cell types and architecture such as the mucus secreting goblet cells, the endocrine cells and M-cells that have been reported to facilitate the transportation of food components across the intestinal barrier and allowing the simulation of the possible hydrolysis by brush border enzymes (Westerhout et al., 2015). Previous studies have demonstrated that at physiological concentrations, the uptake and transportation of carotenoids across the intestinal epithelium is protein mediated (Reboul, 2019). However, other studies indicate that both route and rate of transportation of nutrients are influenced by different factors related to the physiological parameters of the intestinal epithelial tissue. These include such factors as enzyme activity, lipid and protein content of the enterocyte membrane and the amount of Peyer's patches in the tissue (Westerhout et al., 2015). All these are physiological factors that characterise the intestinal epithelium and are practically absent on the differentiated Caco-2 cells. As both Caco-2 cells and Ussing chambers are used to investigate the uptake, transport of nutrients and ultimately predict bioavailability *in vitro*, the bioavailability of carotenoids is relatively low, in the range of 3-34% and varying according to dietary sources and food processing and preparation procedures (Corte-Real et al., 2014). These procedures are part of the rate-limiting step that determine carotenoid

bioavailability from the food matrix during intestinal digestion. Other factors reported to affect the bioaccessibility of carotenoids are outlined in Section **1.5**.

## 1.5 Factors affecting the bioaccessibility and bioavailability of carotenoids

### 1.5.1 Physicochemical properties of carotenoids

Among the major factors that affect carotenoid bioaccessibility is the geometric configuration of the compounds in the food matrix. A study by Sy et al. (2012) investigating the physicochemical properties of carotenoids by comparing the efficiency of incorporation of carotenoids in synthetic micelles with carotenoids naturally incorporated in a food matrix found that in both instances, carotenoids with terminal six-membered rings were more easily transferred to the micellar phase than lycopene (Sy et al., 2012). In terms of cellular accumulation, experimental works by During et al. (2002) found that there was preferential uptake of all-*trans*-β-carotene compared to both 9-*cis*- and 13-*cis*-β-carotene following 16-hour incubation on differentiated Caco-2 monolayer cells. Similarly, all-*trans*-β-carotene was found to preferentially accumulate in human chylomicrons, very-low-density lipoproteins compared to 9-*cis*-carotene (Rodriguez-Amaya, 2015). The authors attributed the observed differences in plasma response of the geometric isomers to selective intestinal transport of all-*trans*-β-carotene at the expense of its 9-*cis* isomer. The importance of molecular structural variables and how these conformations affect carotenoid response in both *in vitro* and *in vivo* studies was also recently revealed (Moran et al., 2018) with all-*trans*-β-carotene reported to be more bioavailable, showing 11% bioavailability for all-*trans*- compared to 2 and 3% for 9-*cis* and 13-*cis* respectively.

### 1.5.2 Food matrix

The subcellular location and type of food matrix in which carotenoids are embedded is another critical factor that affects carotenoid bioaccessibility as these form a natural barrier to external factors that may lead to their degradation. Specifically, the plant organelles (chromoplasts and chloroplasts) are subcellular structures that provide a stable environment preventing carotenoid release and chemical degradation (Raikos, 2017). The role played by these structures was exemplified in a study that found the bioavailability of β-carotene to be affected by chromoplast structure in mature plant tissues, with globular chromoplasts providing the best bioavailability and the crystalline structures

providing the poorest bioavailability of carotenoids (Amorim-Carrilho et al., 2014). This is because carotenoids in globular chromoplasts are found as lipid-solubilised structures unlike the membrane-bound crystalline structures (Jeffery et al., 2012). Likewise, carotenoids in rice food matrix, embedded in chromoplasts, are reported to be more bioaccessible than carotenoids found in green leafy vegetables, where carotenoid pigments are embedded in chloroplast structures (Bechoff and Dhuique-Mayer, 2017). The difference in the bioaccessibility of carotenoids in the two food matrices is due to the high levels of insoluble fibres in the leafy vegetables compared to rice (Bechoff and Dhuique-Mayer, 2017). The extent to which carotenoids in liquid, e.g. puree or pulp, purified form or in the original food matrix affect their bioaccessibility and ultimately bioavailability has equally been investigated thoroughly. In one case, a 2-fold increase in the percentage of carotenoid bioaccessibility was observed from a fruit juice mixture 50.75% (v/v) of papaya, 19.25% (v/v) mango with *Stevia rebaudiana* infusion 2.5% (w/v) compared to unprocessed samples (Barba et al., 2017). Another study assessing food matrix composition was able to demonstrate that bioaccessibility could be determined not only by the cell wall integrity but also by interactions between structural compounds of the complex food matrix. In this randomised, double-blind, crossover, comparative, single-dose study in healthy subjects, the bioavailability of zeaxanthin and lutein was found to be dependent on the formulation used, with the starch-based formulation containing the xanthophylls clearly performing better than alginate-based formulation (Evans et al., 2013; Rodriguez-Amaya, 2015).

### 1.5.3 Processing effects

In plant matrices, carotenoids are often found bound to indigestible polysaccharides, fibres and protein compounds and as such, disruption of these structures through mechanical and thermal processing methods allow for their release and thus accessibility for absorption or extractability (Barba et al., 2017). Food processing can either be categorised into traditional processing technologies such as heat related thermal treatment and novel emerging non-thermal techniques such as high pressure processing, high-intensity pulse electric fields or ultrasound treatments, that are used to preserve manufactured food, as well as during food preparation i.e. pre-treatments on raw material to obtain food (Cilla et al., 2018). Although processing, in particular thermal processing, can significantly reduce carotenoid content in the food matrix, several *in vitro* and *in vivo* studies have shown that it can increase bioaccessibility and bioavailability of carotenoids. For instance, in a study aimed at assessing the difference between industrially processed

and hand-squeezed orange juice on the bioaccessibility of carotenoids, industrial processing of orange juice resulted in reduced particle size and thereby increasing the surface area for the bioaccessibility of carotenoids (Stinco et al., 2012). Hedren et al. (2002) also reported a 100% increase in the release of β-carotene from carrot pieces after heat treatment and 1.3 times in homogenised samples. The general understanding in this case is that thermal processing leads to the softening of the cell structure for the digestive enzymes to efficiently work on the liberated carotenoids from the plant tissue. A three-way crossover study in which participants consumed a homogenised meal resulted in a nine-fold increase in postprandial area under the curve (AUC) levels of β-carotene for the highly homogenised meal compared to the non-homogenised meal (van het Hof et al., 2000).

### 1.5.4 Influence of dietary lipids on carotenoid bioavailability

Lipids are thought to influence the absorption of dietary carotenoids as they promote the release of these compounds from the food matrix by providing a hydrophobic phase in which carotenoids can be solubilised. More so, lipids and their digestion products have been reported to stimulate the secretion of pancreatic enzymes and bile salts thereby facilitating the formation of micelles, including serving as structural components of the mixed micelles (Rodriguez-Amaya, 2015), a feat that increases the uptake of carotenoids. While a plethora of studies have demonstrated how amount and type of dietary lipids is able to influence carotenoid bioavailability (Garrett et al., 2000; Colle et al., 2012; Desmarchelier and Borel, 2017; Chilungo et al., 2019), approximately 5-10 g of fat in a meal is required for efficient absorption of carotenoids (Shilpa et al., 2020). Among the types of fat that have previously been assessed, carotenoid micellarisation has been found to be two to three times higher in dietary fat rich in unsaturated fatty acids (Mashurabad et al., 2017). A recent dietary study revealed that supplementation of green leafy vegetables with olive oil enhanced intestinal accessibility of lutein, reflected as high levels of lutein in plasma and eye samples of aged lutein-deficient mice (Shilpa et al., 2020). The actual mechanism in this case is not entirely clear but one possibility is the presence of oleic acid in the olive oil which could potentially promote the integration and formation of lutein-enriched mixed micelles. Chain length and degree of saturation of dietary triacylglycerols have been reported to affect the bioaccessibility of carotenoids differently. For example, excipient nano-emulsions formulated from medium chain triglycerides were shown to be highly effective at increasing the bioaccessibility of lutein in spinach, whereas long chain triglycerides were required to increase the bioaccessibility

of β-carotene (Yao et al., 2019). Regarding the degree of saturation, evidence suggests that carotenes and xanthophylls also behave differently: carotenes are more bioaccessible in lipids rich in unsaturated fats, while xanthophylls show greater bioaccessibility in the presence of saturated fatty acids (Xavier and Mercadante, 2019). It has been postulated that xanthophylls form smaller mixed micelles sizes. Previous studies report that the smaller sizes of micelles rich in saturated fatty acids increase the amount of xanthophylls that can be incorporated per amount unit of micelle lipids (Gleize et al., 2013). Because of these differences in bioaccessibility, it is important for one to take into consideration the choice of lipid composition as these may have significant implications on carotenoid uptake and subsequent bioavailability.

### 1.5.5 Host-related factors

The absorption efficiency of carotenoids has been found to be variable, falling in the range of 5 and 90% (van Lieshout et al., 2003; Reboul, 2013). This is because the absorption of carotenoids is modulated by such factors as the health and nutritional status of the host. Once provitamin A carotenoids in the diet are absorbed, they are converted to vitamin A via different enzymatic pathways. Depending on the structure of the carotenoid, cleavage can either be through central cleavage catalysed by β-carotene 15,15'-monooxygenase (BCO1) or through eccentric cleavage by β-carotene 9,10' –dioxygenase (BCO2) (Leung et al., 2009). Details of this mechanism are described in section **1.7**. In the case of variable absorption, evidence suggests that plasma carotenoid concentrations decrease substantially in subjects who consume fat soluble components that are not absorbable or absorbable only to a limited extent (van het Hof et al., 2000). For instance, patients presenting with malabsorption syndromes, including inflammatory bowel, celiac disease as well as pancreatic insufficiency from cystic fibrosis, have been found to have lower plasma concentrations of carotenoids (Moran et al., 2018). In the case of nutritional status, it has now been acknowledged that vitamin A status regulates β-carotene absorption and cleavage efficiency via a negative loop (Borel and Desmarchelier, 2017). In this particular scenario, individuals with adequate stores of vitamin A tend to experience lower absorption and cleavage efficiency in comparison to those with deficient stores. Another study found that protein deficiency reduced enzymatic activity of BCO1. Authors reported decreased plasma vitamin A following administration of β-carotene in protein deficient rats compared to the control ones (Shilpa et al., 2020). A recent review further attributed the variability in the absorption efficiency of carotenoids to their moderate uptake and transport through the enterocyte (Reboul, 2019). Considering that protein

transporters are involved in the intestinal uptake of carotenoids, is suggesting that variations in the genes encoding these proteins may modulate carotenoid absorption efficiency. An association study investigating the influence of candidate single nucleotide polymorphism (SNPs) of genes involved in metabolism of lipids on the fasting blood concentration of several carotenoids found that genetic polymorphisms in the scavenger receptor class B member 1 (SCARB1) were associated with β-carotene and not lycopene concentrations (Borel et al., 2007). The involvement of SNPs in the inter-individual variability of carotenoid bioavailability has also been reported in other recent studies (Borel et al., 2014; Borel et al., 2015b; Borel et al., 2015a).

## 1.6 Intestinal absorption of carotenoids

The long held view was that intestinal uptake of carotenoids is by passive diffusion through the gut cells. With this mechanism, micelles in which carotenoids are incorporated migrate through the unstirred water layer to the brush border membrane at which point the carotenoids leave the micellar structure, diffusing through the membrane into the cytoplasm of enterocytes (Yonekura and Nagao, 2007; Rodriguez-Amaya, 2015). However, recent evidence is suggesting that absorption of carotenoids at physiological concentrations is a complex receptor-mediated process. This involves transporters such as scavenger receptor class B type I (SR-BI), cluster of differentiation 36 (CD 36) molecule and Niemann-Pick C1-like1 (NPC1L1) intracellular cholesterol transporter (Desmarchelier and Borel, 2017; Kopec and Failla, 2018; Reboul, 2013). According to Reboul (2019), SR-BI is involved in the intestinal uptake of provitamin A carotenoids, lycopene as well as phytoene and phytofluene. CD 36, a membrane protein highly expressed at the brush boarder level of the duodenum and jejunum, is said to facilitate the uptake of β-carotene, α-carotene, β-cryptoxanthin, lycopene and lutein but not phytoene and phytofluene (Reboul and Borel, 2011; Mapelli-Brahm et al., 2019). Furthermore, NPC1L1, a major sterol transporter in the intestines, has been reported to be involved in the transportation of α-, β-carotene, β-cryptoxanthin and lutein but not lycopene, phytoene and phytofluene (Reboul, 2019; Sato et al., 2012). The possibility of carotenoids crossing the brush border membrane of the absorptive epithelial cells under passive diffusion is particularly certain at supra-physiologic concentrations (Kopec and Failla, 2018). It is in the intestinal cells that provitamin A carotenoids are absorbed intact or metabolised into retinal, retinoic acid and small amounts of β-apocarotenals (Rodriguez-

Amaya, 2015). The actual process of carotenoid conversion into vitamin A is explained in the following section **1.7.**

## 1.7 Bioconversion to vitamin A

Carotenoids are well-known for their provitamin A activity in human health. Indeed, the biological transformation of carotenoids to vitamin A forms the basis for recommendation to consume foods rich in provitamin A carotenoids to maintain vitamin A body stores. In fact, most populations in the developing world depend on plant based diets to meet the body's vitamin A requirements (Harrison and Kopec, 2018). Previous studies have established that the conversion of provitamin A carotenoids to vitamin A (retinol) takes place in the small intestines via enzymatic cleavage of carotenoids by β-C-15,15'-oxygenase 1 (BCO1) enzyme (Burri et al., 2016; Desmarchelier and Borel, 2017; Bohn, 2019; Coronel et al., 2019; Failla et al., 2019; Reboul, 2019). The conversion process in biological systems is known as bioconversion and refers to the proportion of absorbed provitamin A carotenoids that is converted to vitamin A (Rodriguez-Amaya, 2015). Following uptake by the enterocytes, carotenoids are either absorbed intact or converted to vitamin A through enzymatic cleavage of provitamin A carotenoids. Two mechanisms for the cleavage of provitamin A carotenoids have been proposed: central and eccentric ( Figure 1-1) (Rodriguez-Amaya, 2015). Central cleavage is catalysed by BCO1 with cleavage occurring at the 15,15'- double bond yielding at least one molecule of retinal and two molecules from β-carotene (Desmarchelier and Borel, 2017; Failla et al., 2019) in the intestinal cell or liver cytosol. At this stage, retinal can either be reversibly reduced to retinol by retinol reductase or irreversibly oxidised to retinoic acid by retinal dehydrogenase (Olson and Lakshman, 1990). Central cleavage at position 15,15'- double bond only happens in carotenoids with a β-ionone ring, therefore rendering provitamin A carotenoids the only substrates for this enzyme. The route using eccentric or asymmetric cleavage is catalysed by BCO2 at the 9,10'- double bond to yield β-apo-8'-carotenal and β-ionone and takes place in the inner mitochondrial membrane. Eccentric cleavage products are then modified via enzymatic and non-enzymatic reactions (Failla et al., 2019) to form retinol and retinoic acid. The retinol that is formed in both pathways is then esterified by lecithin:retinol acyltransferase (LRAT) generating retinyl esters before incorporation into chylomicrons and secreted into the lymph for use or storage. The incorporation of generated retinyl along with other dietary lipids, takes place in the intestinal mucosa (Vogel et al., 1999).

The conversion of provitamin A carotenoids to retinol has been noted to be affected by factors associated with both bioaccessibility and bioavailability (Furr et al., 2005). For instance, a study assessing carotenoid availability from biofortified sweet potato using Mongolian gerbils demonstrated that the efficiency of bioconversion of β-carotene to retinol was dependent on the fat content of the diet (La Frano et al., 2014). Dietary fat has been found to provide a sink for deposition of carotenoids during the early stages of digestion (Mills et al., 2009). Being lipophilic in nature, carotenoids and other lipid digestion products stimulate the secretion of bile acids that further enhances the formation of mixed micelles. In their study, La Frano et al. (2014) found a bioconversion of 3.5:1 in a high-fat diet compared to 6.5:1 and 6.7:1 for a low-fat diet and control diet respectively. Similarly, the bioconversion of β-carotene to retinol from provitamin A, though varied as it ranged from 2.8:1 to 5.5:1, was found to be more efficient than values reported by the Institute of Medicine (IOM). Vitamin A status has also been reported to be a limiting factor in the bioconversion of provitamin A carotenoids to retinol. In literal terms, conversion to retinol is more efficient in subjects with low vitamin A stores but becomes less efficient as more β-carotene is consumed (Rodriguez-Amaya, 2015), therefore creating a regulatory mechanism for its conversion. Evidence of regulation of β-carotene conversion was documented in a recent study in which doubling the amount of deuterium-labeled β-carotene (from 20 to 40 mg) doubled the area under the curve (AUC) for β-carotene, a measure of absorption, but only increased plasma labeled retinol AUC by 36%, an indication that bioconversion of high doses of β-carotene is regulated (Moran et al., 2018).

Several methods, including animal models, depletion-repletion techniques, oral-faecal balance techniques, postprandial plasma response and the use of stable isotope (van Lieshout et al., 2003) have been used to estimate bioconversion and ultimately, bioavailability of carotenoids. The typical postprandial plasma response involves analysis of the most recent absorbed carotenoids that appear in the triacyl-glycerol rich lipoprotein (TRL) fraction of the plasma. These are rich in chylomicrons and mostly calculated as area-under-time-curve with the peak appearing 10-12 hrs above baseline after test meal intake (Bohn et al., 2018). This estimation on the basis of AUC, translated into fractional absorption is a marker for bioavailability. Of these methods, only isotopic tracer methods are considered to provide required accuracy as the postprandial plasma response is not sufficiently reliable. This is because of the fact that plasma carotenoid response can provide data on the changes of carotenoid concentrations in total plasma but unable to distinguish from recently consumed and already present carotenoids, thus failing to

accurately assess the bioconversion of provitamin A carotenoids to retinol (La Frano et al., 2014). Therefore, the recommended method for assessing the bioconversion of provitamin A to retinol is to use isotope dilution methods because of their ability to appraise the true provitamin A value of dietary carotenoids (Furr et al., 2005).

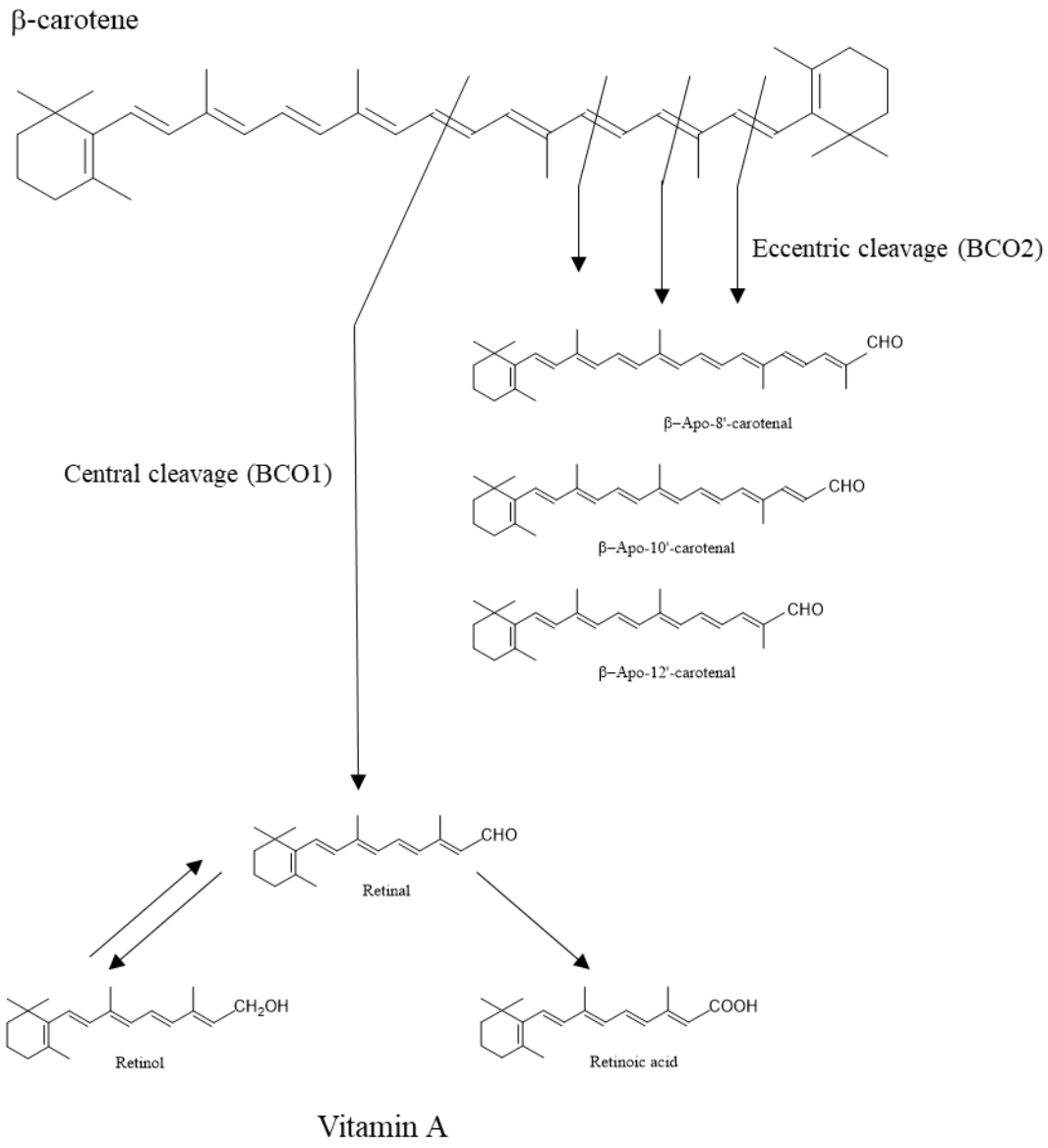



Figure 1-1: Central and eccentric cleavage of β-carotene (adapted from Harrison (2012)).

## 1.8 Bioefficacy and bioequivalence

One of the greatest challenges faced by nutrition scientists today is the ability to quantify the micronutrient value of foods, assess the micronutrient status of individuals, the bioavailability and bioefficacy upon their consumption. This is because scientists are unable to access parts of the body in a non-invasive manner through which a nutrient may travel before appearing in accessible body fluids such as blood or urine (Sheftel et al., 2018). According to van Lieshout et al. (2001), bioefficacy is defined as the efficiency with which the ingested nutrients (in this case, provitamin A carotenoids) are absorbed and converted to an active form of the nutrient (retinol). On the other hand, bioequivalence, also known as conversion factor, is defined as the amount of the converted active form of a nutrient (retinol for this example) obtained from a given dose of a nutrient (provitamin A carotenoid) (Tang and Russell, 2004). This is calculated using the mean vitamin A equivalency of β-carotene, defined as the amount of ingested β-carotene in micrograms that is absorbed and converted into 1 μg retinol (Haskell, 2012). In bioefficacy studies, stable isotope-dilution techniques are used and in most cases utilised to assess the efficacy of an intervention (Sheftel et al., 2018). Previous studies report that about 70% of β-carotene conversions is thought to occur in the intestinal lumen (Bohn et al., 2017). However, using stable isotope techniques, Tang et al. (2003) estimated that only 20-30% of conversion occurs after absorption. Although the β-carotene conversion to retinol is poor, even for pure β-carotene unless the dose is small and fed regularly until equilibration is reached (Rodriguez-Amaya, 2015), bioefficacy studies in human populations are important because of their ability to generate practical results based on host-related factors (Bechoff and Dhuique-Mayer, 2017). In this case, the disease, micronutrient status and inter-individual genetic variations tend to become important determinants in assessing the conversion efficiency. Considering that depletion-repletion methods are used to determine bioequivalence, assumptions based on the theoretical yield of provitamin A carotenoids are critical in estimating retinol activity equivalents (RAE). The first assumption is that, β-carotene in oil as the most bioavailable form, has a ratio of 2:1 to vitamin A *in vivo* while its bioavailability from food is a third of its pure form in oil (Furr et al., 2005; Rodriguez-Amaya, 2015). Therefore, on equivalence terms, one μg retinol translates to 6 μg (2 x 3 μg) of the dietary β-carotene. For α-carotene and β-cryptoxanthin, containing one as opposed to two β-ionone rings found in β-carotene structure, theoretically contribute half of the β-carotene to retinol equivalence i.e. 12 μg. However, previous studies conducted to estimate vitamin A

equivalents from provitamin A carotenoids in populations in developed and developing countries found highly variable mean equivalence ratios ranging from 3.6:1 to 28:1 (Haskell, 2012; Van Loo-Bouwman et al., 2014). According to Haskell (2012), the variations in equivalence ratios could be attributed to differences in experimental designs, differences in response indicators for β-carotene and retinol, nutritional status and polymorphism related to β-carotene metabolism. For this reason, accurate testing tools and standard procedures should be given priority consideration as they have practical implications on both the true vitamin A value of diets and during planning and design of effective dietary interventions.

## 1.9 Choice of study material

Given that plant food sources account for more than 80% of the vitamin A requirements in individuals in areas most afflicted with VAD, consumption of foods rich in provitamin A carotenoids has been reported as a sustainable strategy likely to improve the vitamin A status in humans (Blomme et al., 2020). In the present experiment, different food materials were selected for specific purposes. For instance, goji berries, kale and butternut squash (BNSQ) used in chapter 4 were carefully selected as they provide distinct structural, energy and carotenoid composition differences which would impact on both digestion and nutrient absorption. Biofortified maize (*Zea mays*) and sweet potato (*Ipomoea batatas*) on the other hand, produced as cereal and root crops respectively, were chosen as they are staple foods consumed by over a billion people globally applying different processing methods (Chitchumroonchokchai et al., 2017). Through biofortification, they constitute excellent sources of provitamin A carotenoids in the human diet and therefore, their consumption is being promoted world over. Particularly, sub-Saharan African countries are using these as a food-based strategy to complement existing efforts in reducing the occurrence of VAD (Burri et al., 2011; Gannon et al., 2014; Bouis and Saltzman, 2017; Chitchumroonchokchai et al., 2017; Awuni et al., 2018). The underlying assumption is that sustained consumption of these nutrient dense staple crops can substantially reduce micronutrient deficiencies through daily adequacy of dietary intakes among individuals throughout the life cycle (Bouis et al., 2011). While consumption of nutrient enhanced foods holds the promise for sustainable and cost-effective food-based solutions to combat micronutrient deficiencies (Gupta et al., 2015a), of major concern however is the fact that the carotenoids in these staple crops are highly susceptible to postharvest degradation when exposed to heat, oxygen and light owing to

their unsaturated structures (De Moura et al., 2015). This is especially true when foods are prepared differently and even more so, as composite meals. Secondly, composite meals are ordinarily consumed in a plethora of combinations and as such, come in different structures and compositions. In this study, the composite meal was made up of *Nshima* from maize meal and stew prepared under traditional methods as described in **section 2.2.2**. *Nshima* is the local name for cooked grain meal that is consumed as a staple food in Zambia. Prepared as a stiff porridge, *Nshima* is often eaten with fish, meat or stew and some form of vegetable soup. Its equivalent in South Africa is called *Phutu* (Pillay et al., 2014), *Sadza* in Zimbabwe and *Ugali* in East Africa (Muzhingi et al., 2008). Recent data suggests food structural features, specifically composition, meal size and caloric content have the ability to alter gastric behaviour and therefore impact on nutrient delivery and digestion in the small intestines (Mulet-Cabero et al., 2017; Verhoeckx et al., 2015). Data on the kinetics involved with which gastric digestion affects delivery and bioaccessibility of dietary carotenoids from test foods with varied calories is however poorly understood. Lastly, for dietary carotenoids to exert their health and nutritional potential, they need to be released from the food matrix during GI digestion, solubilised and taken by the enterocyte cells and made available for physiological functions and storage within the human body. Unfortunately, evaluation of carotenoid bioavailability has long been hampered by the limited knowledge of their absorption mechanisms as well as limitations presented by both *in vitro* and *in vivo* experimental approaches (Yonekura and Nagao, 2007).

## 1.10 Study hypothesis and research objectives

The main hypothesis of this research work was that processing and food preparation methods would improve the bioaccessibility of carotenoids in biofortified test foods.

The research objectives are chapter specific and are outlined as follows:

I. To investigate the content, retention and stability of dietary carotenoids in different varieties of biofortified foods as affected by processing, preparation and storage conditions (**Chapter 3**).
II. To assess the impact of food structure and composition on carotenoid bioaccessibility in composite meals prepared from biofortified foods as affected by caloric density and gastric digestion kinetics (**Chapter 4**).
III. To determine the fate of dietary carotenoids in biofortified foods following simulated GI digestion and exposure on differentiated Caco-2 cells and mouse intestinal tissue (**Chapter 5**).

# CHAPTER 2

# Materials and Methods

This chapter describes chemicals and reagents, food and test samples, preparation of solutions and reagents, general equipment and analytical techniques that apply to more than one chapter. Specific methods and techniques are described in individual chapters.

## 2.1 Materials

### 2.1.1 Chemicals and reagents

Only chemicals and reagents of analytical and HPLC grade were used in the present project. Extraction chemicals sodium chloride, butylated hydroxytoluene (BHT), potassium hydroxide and crystalline ammonium acetate were obtained from Sigma-Aldrich (Dorset, UK) as well as solvents used during extraction such as ethanol, 1,2-dichloroethane, 2-propanol and hexane. Analytical solvents methanol and methyl tert-butyl ether (MTBE) were obtained from Fisher Scientific (Loughborough, UK). High purity carotenoid standards (>95%): *all-trans*-β-carotene and β-apo-8'-carotenal were purchased from Sigma-Aldrich; lutein, zeaxanthin and β-cryptoxanthin came from Extrasynthese (Lyon, France). All chemicals and products used in the preparation of artificial micelles and enzymes used during simulated digestion procedures were purchased from Sigma-Aldrich while sodium taurocholate was obtained from Fisher Scientific. Unless otherwise stated, all cell culture medium components were purchased from Gibco (Life Technologies, Warrington, UK).

### 2.1.2 Food and test samples

Butternut squash (*Cucurbita moschata*), sweet potato (*Ipomoea batata*) and white maize (*Zea mays*) were purchased from Kirkgate open market in Leeds, UK. Kale and goji berries were purchased from Morrison's supermarket in Leeds. Five (5) varieties of biofortified Orange-fleshed sweet potato (OFSP) (*Kakamega, Lunga, Yellow Santura, Chumfwa* and *Chingovwa*) were a kind donation from Zambia Agriculture Research Institute (ZARI) while four (4) varieties of biofortified orange maize (GV 662, GV 664, GV 665 and GV 672) were a gift from International Centre of Tropical Agriculture – HarvestPlus (CIAT-HarvestPlus) Zambia. Samples of the newly developed chickpea varieties were a donation by Dr Jana Kholova from the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT) in India while Dr Gabriela Toledo-Ortiz

from Lancaster University, through N8 AgriFood collaboration donated tomatoes originating from Mexico for carotenoid content analysis. These samples were analysed using a method that was optimised with food material purchased from Kirkgate open market in Leeds, UK. The overview of the steps taken during sampling, processing and analysis of study material is as shown in Figure 2-1.

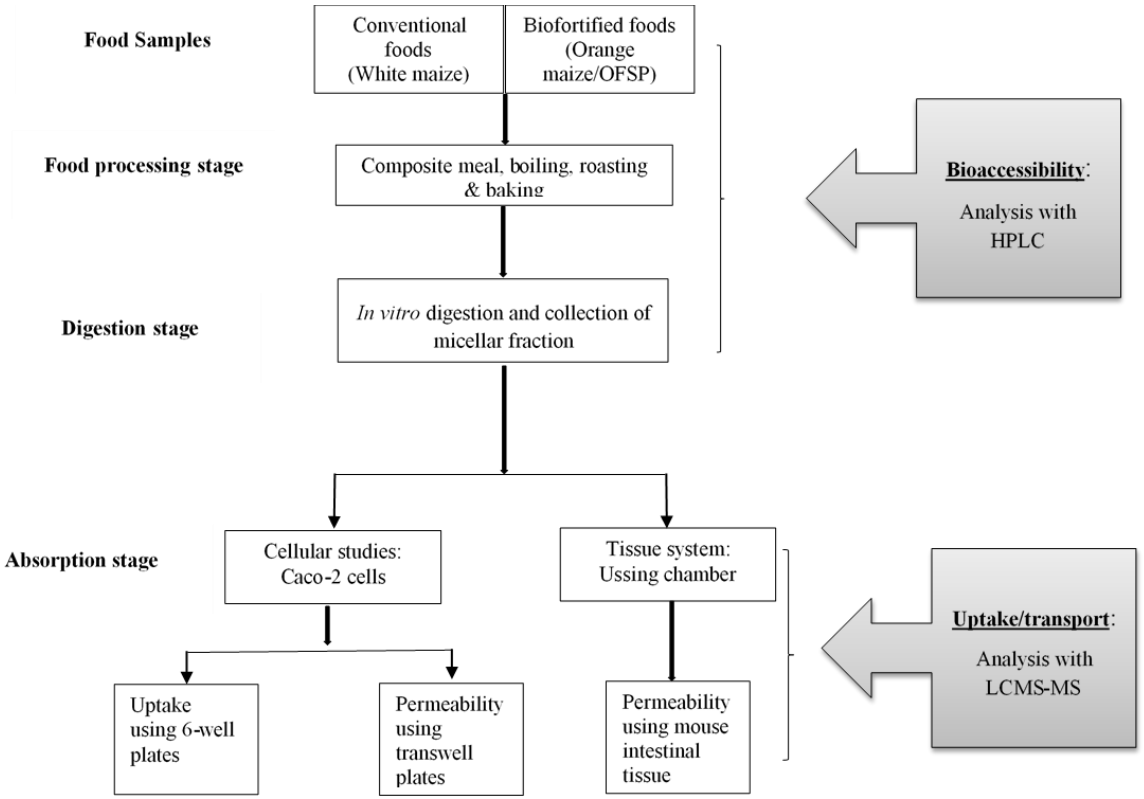



Figure 2-1 Overview of key parameters used in the processing and analysis of dietary carotenoids in test foods.

### 2.1.3 Preparation of HPLC reagents

Unless stated, all aqueous solutions used in the analysis of carotenoids using the HPLC were prepared using ultrapure, nuclease-free water (≥ 18.2 mΩ cm at 25°C) from a Millipore water purifying system. Ethanol containing 0.1% (w/v) butylated hydroxytoulene (BHT) and methanol containing 0.01% (w/v) BHT kept at room temperature (25°C) for no more than one week. Similarly, HPLC grade methanol in equal volumes with 1,2-dichloroethane, 80% (w/v) potassium hydroxide in absolute ethanol, and hexane containing 0.01% BHT were prepared, stored at room temperature for a maximum period of one week. MTBE (>95 %, Chromasolv HPLC grade) was filtered using 0.22 µm PTFE filters prior to use.

### 2.1.4 Carotenoid standard stock solutions

Carotenoids (*all-trans*-β-carotene, β-cryptoxanthin, lutein and zeaxanthin) previously stored at -20°C were brought to room temperature. Stock solutions (0.5 mg/mL) were prepared by dissolving carotenoids in appropriate solvents and working concentrations determined spectrophotometrically using published wavelengths and extinction coefficients (Britton et al., 2004). Briefly, to each carotenoid, 1 mL of 2-propanol was added in a 20 mL glass test tube, mixed and after complete dissolution, another 4 mL of 2-propanol was added to bring the total volume to 5 mL. From each 20 mL glass tube of the carotenoids, 100 µL was transferred to a new 20 mL glass tube and 3 mL of 2-propanol, 3 mL hexane and 1 mL Milli-Q water added, mixed and vortexed for 1 minute. In the resulting dispersion with two distinctly separated layers of carotenoid containing hexane layer and 2-propanol water layer, the upper layer (hexane/carotenoid) of approximately 3 mL was transferred to a new 20 mL glass tube with a glass Pasteur pipette and dried under a steady flow of nitrogen gas. The dried carotenoids were then reconstituted in 2 mL methanol:1,2-dichloroethane (50:50), mixed and vortexed for 1 minute. The dissolved carotenoids were then filtered through 0.22 µm PTFE filters before reading the absorbance on the spectrophotometer at 450 nm.

### 2.1.5 Preparation of β-apo-8'-carotenal recovery standard

Approximately 1 mg of β-apo-8'-carotenal was weighed and placed in a screw-capped 15 mL glass tube and 3 mL of 2-propanol, 3 mL hexane and 1 mL deionised water added, mixed and vortexed for 1 minute. In the resulting solution with two distinctly separated layers, carotenoids were extracted following the procedure described in section 2.1.4. The absorbance of β-apo-8'-carotenal was adjusted to ~0.6 as a working standard and kept at -20°C for 1 week.

## 2.2 Methods

### 2.2.1 Sample preparation

For maize, between 50 and 60 maize grains from a representative sample of a maize genotype were selected. These were ground to a fine flour using a coffee grinder (KRUPS, UK) and sieved though a 500 micron sieve (Endecotts, UK) to obtain a fine powder with particle size < 500 µm, ensuring that at least 90% of the grounded powder was sieved. The flour was then packaged into 50 g portions and kept at -80°C until the day of analysis.

Sweet potato was chopped into small cylindrical shapes and divided into 3 processing methods as follows;

- A third of what was chopped immediately kept at -20°C for 48 hours before freeze-drying and final storage at -80°C until analysis.
- Second third was either boiled or baked, kept at -20°C for 48 hours before freeze-drying and final storage at -80°C until analysis.
- Last third was immediately used in the preparation of composite meals after which half was analysed and the other half stored at -80°C until analysis.

All manipulations were done under appropriate dim lighting to reduce photo degradation of carotenoids resulting from direct UV light.

### 2.2.2 Meal preparation

Composite meal preparation from maize flour was done according to previously described protocols (Mugode et al., 2014; Pillay et al., 2014) with modifications while boiling and baking procedures on OFSP were standardised following procedures described by Bengtsson et al. (2008). For *Nshima meal* (described in section 1.9), prepared from both non-biofortified and biofortified maize meal, 150 ± 5 g of maize flour was used with 500 mL of tap water. Briefly, 75 g of maize flour was slowly added with continuous stirring to water previously warmed to 50°C with a wooden spoon until formation of a thick gel. To ensure thorough cooking of the added maize flour, boiling continued for a further 15 minutes after which the remaining 75 g of maize flour was added with continuous stirring until a thick paste was formed. The final *Nshima* was then placed on a plate to cool before mixing it with previously prepared chicken stew (from lean breast chicken) containing 5 and 10 % of vegetable cooking oil (w/w). The proportion of chicken stew to *Nshima* was also prepared into two different compositions of 5 and 10 % on a w/w percent basis with *Nshima* to make *Nshima meal*. Small cylindrical shapes of OFSP weighing 200 g in total were added to 500 mL boiling water (ca. 100°C) and cooked

at a steady rolling boil for 15 minutes after which the food was allowed to cool to 50°C before mashing with standard kitchen utensils. The mashed potato was then mixed with previously prepared 5 or 10 g (w/w) lean breast chicken (representing protein) containing 5 and 10 % of vegetable cooking oil on a w/w percent basis. The food groups and their method of preparation is as described in Table 2-1. All samples were then vacuum-sealed in black plastic bags, labelled and stored at -80°C.

Table 2-1: Methods of food preparation

| Food group | Ingredients | Preparation method |
|---|---|---|
| Kale | Kale leaves, onion, tomato, vegetable oil and salt | Bunch of chopped kale leaves stir-fried in vegetable oil with chopped onion and tomato in aluminium pot at medium heat for 10-12 min |
| OFSP | OFSP | Chopped OFSP boiled in 500mL in aluminium pot and boiled for 15 min at high temperature (ca.100°C) |
| BNSQ | BNSQ | Chopped BNSQ boiled in 500mL in aluminium pot and boiled for 15 min at high temperature (ca.100°C) |
| *Nshima meal* (Composite) | Cooked *Nshima* and chicken stew | Cooked *Nshima* mixed with different proportions (5 or 10% w/w chicken stew) |
| OFSP meal (Composite) | Boiled OFSP and beef stew | Boiled OFSP mixed with different proportions (5 or 10% w/w chicken stew) |
| Chicken stew | Lean chicken breast, onion, tomato, vegetable oil, water and salt | Chopped chicken pieces boiled in vegetable oil with chopped tomato and onion in aluminium pot at medium heat (ca. 75°C for 25-30 min |
| *Goji berries | Nil | Grounded in mortar and pestles under liquid nitrogen |

*Did not undergo any form of heat/thermal processing but only grounded to powder. OFSP (Orange fleshed sweet potato); BNSQ (Butternut squash).

### 2.2.3 Determination of caloric content and gastric emptying

Food composition and caloric density in particular, have been found to have a direct effect on the physicochemical parameters of the food digesta that eventually affects stability, bioaccessibility, gastric emptying and nutrient absorption (Courraud et al., 2013; Hunt and Stubbs, 1975; Mackie et al., 2013; Moore et al., 1984; Mulet-Cabero et al., 2020b; Mulet-Cabero et al., 2017). The amount of food digesta that leaves the stomach into the small intestines during gastric digestion, known as gastric emptying, is dependent on the caloric

content of the test food to be digested i.e. calories per gram of food. Ordinarily, details on caloric content can be found on the packaging material of commercial foods. However, in the absence of such information, caloric content can be estimated through theoretical calculations by knowing the composition of protein, lipid and carbohydrate in the test food and by applying the Atwater factors where 1 g of lipid yields 9 kcal, 1 g protein yields 4 kcal and 1 g carbohydrate yields 4 kcal. Using these factors and taking OFSP composite meal as an example with a dry weight of 4 g and the following nutrient composition; 20.6% carbohydrate, 0.15% fat and 2.01% protein from a simulated 350 g meal size, results in the energy content of 0.263 kcal/mL. This value was calculated on the assumption that 20 g test food will simulate a 350 g meal size. Therefore, a scale of 20 g/ 350 g meal size = 0.06 calculated rate is derived. Considering an average emptying rate of 2 kcal/min (Hunt and Stubbs, 1975) and density set at 1 g/cm$^3$, a 20 g test food sample with a dry weight of 4 g and calorie density of 0.263 kcal/g would empty at a rate of 2 kcal/min * 0.06 = 0.12 kcal/min. As both oral and gastric secretions are taken into account, the volume entering the gastric phase from the oral phase (20 g test food + 4 mL oral secretions) comes to 24 mL giving a total of 48 mL to be emptied. A 20 g test food with a calorie density of 0.263 kcal/g (20 g * 0.263 kcal/g) results in the total energy of 5.26 kcal. Since the caloric emptying rate has been calculated as 0.12 kcal/min, the time taken to empty the gastric phase will therefore be (5.26/0.12) ~ 44 mins and the total emptying rate of (48 mL/44 mins) 1.09 mL/min. As the decision is to sample at four (4) different stages, then each stage is sampled with a volume of (48 mL/4) 12 mL taking place every (44 min/4) 11 mins.

### 2.2.4 Freeze drying of samples

Chopped and previously frozen sweet potato samples and Nshima meal prepared with lipid (vegetable oil) and protein (chicken stew) in the ratio of 85:5:10 and 85:10:5 (w/w) were freeze-dried using a Christ Gamma freeze dryer (SciQuip, Newtown, UK) with a vacuum system set at 0.040 mbar and the condenser at -50°C for 72 hours. All samples were weighed before and after freeze drying.

### 2.2.5 Moisture content determination

For all processed food samples, moisture content was estimated on the basis of dry matter using the gravimetric oven drying method (AOAC, 2005). For this procedure, 5 g of sample was weighed and dried in a convection oven (FEV18) set at 105°C for 4 hours and cooled to

constant weight. The difference before and after drying was considered as water content in the sample. Results were expressed as % moisture.

## 2.3 Simulated gastro-intestinal digestion

### 2.3.1 Enzymes, chemicals and reagents for *in vitro* digestion

The preparation of enzymes, chemicals as well as electrolyte simulated salivary fluid (eSSF), electrolyte simulated gastric fluid (eSGF) and electrolyte simulated intestinal fluid (eSIF) prepared at 1.25X concentration was an adaptation of the procedure described by Minekus et al. (2014) with minor modifications. Mixing of the electrolyte solutions was done in proportions as presented in Table 2-2. The preparation of electrolyte stock solutions at higher concentration was meant to balance the electrolyte ionic composition after addition of enzymes and calcium chloride in the final simulated fluid concentrations.

Table 2-2 Simulated digestion fluids stock solutions prepared to 400 mL volume at 1.25X concentration and stored at -20°C.

| | | | SSF | | SGF | | SIF | |
|---|---|---|---|---|---|---|---|---|
| | | | pH 7 | | pH 3 | | pH 7 | |
| Constituent | Stock | conc. | Stock | Conc. | Stock | Conc. | Stock | Conc. |
| | **$gL^{-1}$** | **mol $L^{-1}$** | **mL** | **mmol $L^{-1}$** | **mL** | **mmol $L^{-1}$** | **mL** | **mmol $L^{-1}$** |
| KCl | 37.3 | 0.5 | 15.1 | 15.1 | 6.9 | 6.9 | 6.8 | 6.8 |
| $KH_2PO_4$ | 68 | 0.5 | 3.7 | 3.7 | 0.9 | 0.9 | 0.8 | 0.8 |
| $NaHCO_3$ | 84 | 1 | 6.8 | 13.6 | 12.5 | 25 | 42.5 | 85 |
| NaCl | 117 | 2 | - | - | 11.8 | 47.2 | 9.6 | 38.4 |
| $MgCl_2(H_2O)_6$ | 30.5 | 0.15 | 0.5 | 0.15 | 0.4 | 0.1 | 1.1 | 0.33 |
| $(NH_4)_2CO_3$ | 48 | 0.5 | 0.06 | 0.06 | 0.5 | 0.5 | - | - |

For the electrolytes prepared in Table 2-2, addition of $CaCl_2(H_2O)_2$ was only made to the final mixture of the simulated digestion fluids and food to avoid precipitation.

### 2.3.2 Enzyme activities

Enzyme activity assessment of α-amylase, pepsin and pancreatin (based on trypsin activity) was performed according to the supplementary material of the protocol by Minekus et al. (2014).

### 2.3.3 Human salivary α-amylase test procedure

Considering that the test samples used in the present experiment (OFSP and Maize) have high starch content (Kitahara et al., 2017), it was imperative that the study established optimal α-amylase activity for use during oral digestion phase. Up to 50% of starch has been reported to

be hydrolysed by the enzyme α-amylase despite the relatively short retention time in the mouth (Minekus et al., 2014).

The α-amylase activity is based on soluble starch assuming that one unit liberates 1.0 mg of maltose equivalent from starch in 3 min at pH 6.9 at 20°C.

Prior to use, 1 unit/mL of α-amylase was prepared in sodium phosphate buffer (pH 6.9) and kept on ice. The substrate (1%, w/v of potato starch) was placed in a 15 mL test tube and incubated at 20°C for 3-4 min. To this, different volumes of enzyme solutions (50, 70 and 100 µL) were added, mixed and incubated at 20°C for 3 minutes. After this time period, the reaction was stopped by adding 100 µL of the 96 mM 3,5-Dinitrosalicyclic acid (DNSA) with sodium potassium tartrate solution (colour reagent solution) to each tube. The procedure was repeated with a second addition of α-amylase, boiled at 100°C for 15 minutes and immediately placed on ice. After this, 900 µL of Milli-Q water was added to the tube and solution mixed by inversion. The reaction mixture was then pipetted to a microtiter plate at 200 µL per well in triplicate and absorbance read at 540 nm using a Biotek plate reader.

Following preparation of the standard curve with different concentrations of maltose solution (0.2, 0.1, 0.08, 0.06, 0.04, 0.02, 0.005 and 0%, w/v) diluted with sodium phosphate buffer (pH 6.9). The blank used in this experiment was the substrate and sodium phosphate buffer without addition of an enzyme. Activity of α-amylase was estimated by extrapolation of the standard curve using the following equations:

**Standard Curve**:

$\Delta A_{540}$Blank corrected absorbance = $\Delta A_{540}$Standard - $\Delta A_{540}$Std.Blank Equation 2-1

Standard curve was established by plotting the $\Delta A_{540}$nm of the standards vs the quantity of maltose (mg) using the following linear equation:

$\Delta A_{540}$Standard = a X [Maltose] + b Equation 2-2

**Enzyme activity**:

$\Delta A_{540}$Sample = $\Delta A_{540}$Test - $\Delta A_{540}$Test Blank Equation 2-3

$$\frac{Units}{mg\ powder} = \frac{[A540\ Test - A540\ Test\ Blank] - b}{(a\ X\ x)}$$ Equation 2-4

Where;

'a' is the slope, 'b' is intercept and 'x' is the quantity of the amylase powder (mg).

### 2.3.4 Pepsin activity measurement

The pepsin activity is based on the ability of haemoglobin substrate to produce a $\Delta A_{280}$ unit change of 0.001 per min when measured at 37°C under acidic conditions (pH 2).

Porcine pepsin (10 mg) was dissolved in 5 mL 10mM Tris buffer and adjusted to pH 6.5 with 150 mM NaCl. Just before the assay, pepsin was diluted in 10 mM hydrochloric acid (HCl) and diluted to the following concentrations: 5, 10, 15, 20, 25 and 30 µg/mL, and placed in 2 mL Eppendorf tubes.

To measure pepsin activity, 500 µL of the 2% haemoglobin solution was pipetted into 2 mL Eppendorf tubes. These were then incubated at 37°C for 3-4 minutes for them to reach temperature. To this was added 100 µL of pepsin assay solution for each concentration except blanks containing Tris buffer and incubated at 37°C for 10 minutes. The reaction was stopped by adding 1 mL of 15% trichloroacetic acid (TCA) to every tube including the blank. To precipitate the haemoglobin, tubes were centrifuged at 6000 x *g* for 30 minutes. The supernatant from each tube was removed and incubated at 20°C until temperature equilibration. The absorbance for each supernatant was then read at 280 nm in quartz cuvettes $A_{280}$.
To ensure that activity was within the linear part of the evolution of pepsin concentration, it was verified that the activity obtained was the same for each tested concentration of pepsin using the following equation:

$$\frac{Units}{mg} = \frac{[A280\ Test - A280\ Blank]}{(\Delta t\ x\ X)} \qquad \text{Equation 2-5}$$

Where
'Δt' is the duration of the reaction, i.e. 10 minutes and 'X' is the concentration of pepsin powder (µg/mL).

### 2.3.5 Pancreatin based on trypsin activity

The principle of trypsin activity is based on its ability to hydrolyse 1 µmol *p*-toluene-sulfonyl-L-arginine methyl ester (TAME) per min at 25°C and pH 8.1.
A 46 mM Tris-HCl buffer volume of 1.3 mL at pH 8.1 was pipetted into a quartz cuvette followed by addition of 150 µL of 10 mM TAME. This was incubated in a spectrophotometer that was previously set at 25°C for 3-4 minutes for temperature equilibration. To this was added 50 µL of the trypsin solution (except the blank) and mixed by inversion. The absorbance was read and recorded at 247 nm during 10 minutes, measured at intervals of 10 seconds.

The plot of absorption versus time in minutes was plotted and the slope (ΔA247) determined from the initial linear part of the curve. The slope $\Delta A_{247}$ [Unit absorbance/minute] for both the blank and test reactions were calculated using the maximum linear rate and over at least 5 minutes using the following equation:

$$\frac{Units}{mg} = \frac{(\Delta A247\ Test - \Delta A247\ Blank) x\ 1000\ x\ 1.5}{(540\ x\ X)} \qquad \text{Equation 2-6}$$

Where;

$\Delta A_{247}$: Slope of the initial linear portion of the curve, [Unit absorbance/minute] for the Test (with enzyme) and Blank

540: Molar extinction coefficient (L/(mol x cm) of TAME at 247 nm

1.5: Volume (in mL) of the reaction mix (Tris-HCl + TAME + Enzyme)

X: Quantity of trypsin in the final reaction mixture (quartz cuvette) (mg).

### 2.3.6 *In vitro* digestion procedure

The simulated *in vitro* digestion of test foods in the present experiment was carried out according to a combination of protocols as proposed by the COST Action INFOGEST network and the recently developed semi-dynamic digestion models (Minekus et al., 2014; Mulet-Cabero et al., 2020a) with adaptations for carotenoid analysis (Rodrigues et al., 2016). Briefly, using the static *in vitro* digestion model, 5 g of the test food was minced in the electric mincer and mixed with 3.5 mL of SSF, 0.5 mL of α-amylase prepared in SSF to achieve a final concentration of 75 U/mL, and $CaCl_2$ at a final concentration of 0.3 mM in a 50 mL brown and low light permitting centrifuge tube. Water (0.975 µL) was added to achieve a 50:50 (v/v) final ratio of food to SSF. The mixture was then incubated at 37°C for 2 minutes to simulate oral transit time. A blank test tube was included in the experiment that had water in place of the test food but contained all the other fluids and enzyme.

The test food (10 mL) from the oral phase was then exposed to the gastric phase where the pH was adjusted to 3 using 1 M HCl and 10 mL of previously warmed (at 37°C) SGF containing porcine pepsin and $CaCl_2$ in final concentrations of 2000 U/mL and 0.75 mM respectively. The sample was then incubated at 37°C in a shaking incubator set at 100 rpm for 2 hours. Prior to this, a test experiment was conducted to determine the amount of acid required for use during digestion. Upon completion of the incubation period of 2 hours at 37°C, the gastric sample-chyme was delivered to the intestinal phase and mixed with pancreatic suspension from porcine pancreas (assessed as trypsin activity), $CaCl_2$ and bile salts prepared in SIF to achieve final

concentrations of 100 U/mL, 0.3 mM and 10 mM in the final mixture. The concentration of bile acid from the extract was ascertained using an enzyme assay (bile acid kit, ref 1 2212 9990 313, DiaSys Diagnostic System GmbH, Germany) and using the manufacter's protocol. The pH of the final mixture was adjusted to 7 in order to neutralise the mixture and addition of water to dilute the SIF stock and also achieve 50:50 ratio of the gastric sample-chyme to SIF. Incubation for the intestinal digestion was set for 2 hours at 37°C on the shaking incubator at 100 rpm. After intestinal digestion, the digesta was placed on ice and centrifuged (Beckman Coulter, UK) at 35,000 x *g* at 4°C for 68 minutes. The centrifugation process allowed a 3-phase separation composed of the oil droplets and other suspensions on top, followed by the aqueous micellar fraction on the middle and the residual solids and sediments forming a pellet at the bottom of the centrifuge tube. Separation of the micellar fraction containing the bioaccessible carotenoids from the non-bioaccessible aqueous fraction was achieved using 13 mm cellulose regenerated syringe filters of 0.22µM pore size (Whatman®, Spartan, UK). An aliquot of the filtered aqueous phase containing mixed micelles was transferred to a foil-covered 15 mL glass test tube, flushed with nitrogen gas and stored at -80°C until further analysis.

### 2.3.7 Carotenoid bioaccessibility

Carotenoid bioaccessibility, referred to as the fraction of carotenoids released from the food matrix during digestion that is potentially available for absorption was calculated as the ratio between the concentration of the carotenoid in the micellar aqueous phase (supernatant) and its initial concentration in the test food (Rodrigues et al., 2016) according to the following equation;

$$\text{Bioaccessibility (\%)} = \left( \frac{[Carotenoid]Supernatant}{[Carotenoid]Test\ food} \right) \times 100 \qquad \text{Equation 2-7}$$

### 2.3.8 Artificial micelle preparation

It has been demonstrated through experimental procedures that absorption of dietary carotenoids in humans comprises of two critical steps – the first step involving the disruption of the food matrix and the second involving solubilisation of carotenoids in the mixed micelles (micellarisation). Solubilisation is one critical step that determines what is taken up by the enterocytes, converted to retinol and subsequently secretion into the lymph (Van Loo-Bouwman et al., 2014). However, the major limitation for the release and solubilisation of these compounds is the matrix in which these compounds are embedded. To assess the food matrix effect and model suitability, artificial micelles were prepared for uptake and transport studies.

The preparation of artificial micelles follows the procedure described elsewhere (Dhuique-Mayer et al., 2007). Briefly, 1-oleoyl-rac-glycerol (0.3 mM), oleic acid (0.5 mM), phosphatidylcholine (0.04 mM), lysophosphatidylcholine (0.16 mM) and cholesterol (0.1 mM) were dissolved in chloroform/methanol (2:1, v/v). To this mixture, *all-trans*-β-carotene and lutein (20 μM for each) dissolved in 15 mL glass tubes containing hexane was then evaporated to dryness under a flow of nitrogen gas. The dried residue was later reconstituted in Dulbecco' modified eagle medium (DMEM) containing 5 mM sodium taurocholate and incubated at 37°C for 30 min. The solution was then sonicated in a sonicator bath (Deco – FS100, UK) to dissolve the solid particles for 30 min and later incubated at 37°C for 1 hour to ensure complete solubilisation. After this incubation period, the solution was filtered through 13 mm cellulose filters (Whatman®, Spartan). Aliquots of the resulting solutions were flushed with nitrogen gas and stored at -20°C to be used within 5 days.

## 2.4 Absorption models

### 2.4.1 Caco-2 cell line

Human colon adenocarcinoma Caco-2 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury, UK). These were routinely maintained in 75 $cm^2$ plastic flasks (Corning®, Corning Inc., UK) in a $CO_2$ incubator (Sanyo, UK) at 37°C and 5% $CO_2$. The cells were grown in DMEM (Gibco, UK) containing 4.5 g/L glucose. The medium was supplemented with 10% FBS (Gibco), 1% non-essential amino acid solution (NEAA, Gibco) and 1% penicillin-streptomycin (Sigma Aldrich).

Cells were routinely passaged when reaching 80% confluence. Medium was aspirated and the cells washed with phosphate buffered saline (PBS) before adding trypsin. After incubation for 5-10 min when the cells were detached, full medium was added to stop enzyme activity. A cell counter (Cyto, Corning, UK) was used to count viable cells.

### 2.4.2 Cellular uptake studies

For cell experiments, cells at passages 15-20 were used and seeded flat-bottomed 6-well plate (Corning®) at a density of 5.0 x $10^4$ cells/$cm^2$. To differentiate Caco-2 cells, they were continuously grown for 21 days and the culture medium changed every second day. On the day of the experiment, the medium was removed and cells treated with carotenoids either from the micellarized fraction post digestion or artificial micelles. Micelles generated from the

simulated digestion were diluted in DMEM in the ratio 1:2 to avoid the toxic action of bile salts on the cells. Cell cytotoxicity was assessed as indicated in section 2.4.5. Cells treated with micelles containing carotenoids were then incubated for 4 hours at 37°C and 5% $CO_2$. After the incubation period, the plates were placed on ice and apical medium removed with the glass Pasteur pipette (Sigma-Aldrich, UK) connected to an automatic aspirator (Grant Bio, UK). Cells were then washed with 1 mL of Dulbecco's Phosphate Buffer Saline (DPBS) to remove the buffer, safely discarded before scraping them in the presence of 0.5 mL ice-cold DPBS.

### 2.4.3 Cell permeability studies

To assess the cell permeability of carotenoids, Caco-2 cells were grown on semi-permeable membrane inserts (Transwell® 0.4 µm, polyester) Transwell® plates at a density of 5.0 x $10^4$ cells/$cm^2$ on 6-well plates. The Transwell® plates allow the transport of compounds from the apical chamber through the cells to the basolateral chamber. Transport studies were then performed on differentiated cells after 21 days of incubation. At the beginning of each experiment, the apical side of the cell monolayer received 2 mL of either artificial micelles containing equimolar concentrations of lutein and β-carotene (0-20 µM) or of physiological micelles generated from orange fleshed sweet potato digesta while the basolateral chamber received a similar volume of normal medium. Cell monolayers on the transwell plates were then incubated at 37°C, 5% $CO_2$, for 16 hours, the period during which chylomicron production is expected to be complete and also allowing maximum absorption and accurate measurement of carotenoids in the basolateral chamber (Reboul et al., 2005; Desmarchelier et al., 2017). After the incubation period, medium was collected from both the apical and basolateral chambers for analysis, together with the cells on the transwell inserts that were harvested by scrapping. The extraction followed the procedure described in Section **2.4.12** with the resulting extract stored at -80°C until Liquid Chromatograph Mass Spectrophotometry (LCMS) analysis.

### 2.4.4 Cell integrity measurements

The integrity of the Caco-2 cell monolayer grown on the semi-permeable membrane of the transwell plate was evaluated using the Trans-epithelial Electrical Resistance (TEER, Millicell®ERS-2, UK) voltohmmeter. TEER, a non-destructive method, is a widely accepted quantitative technique used to measure the tight junction dynamics in cell culture models for epithelial monolayers (Srinivasan et al., 2015). TEER values above 100 Ω.$cm^2$ following subtraction of the background electrical resistance on differentiated Caco-2 cell monolayers is considered satisfactory (Hidalgo et al., 1989).

### 2.4.5 Cell viability assay

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was based on the protocol by Han et al. (2019) with some minor modifications. MTT stock (5 mg/mL) was prepared in complete medium out of which an MTT working solution (0.5 mg/mL) was used for the experiment. The Caco-2 cells were seeded at 5.0 x $10^4$ cells/$cm^2$ on a 48-well plate (Corning®, UK) and incubated at 37°C, 5% $CO_2$ for 48 hours to allow for cell adherence to the plate and reaching 90% confluence. Individual carotenoids were prepared using the 'Tween 40' (200 mL Tween 40/L acetone) method as described previously (O'Sullivan et al., 2007) and concentrations determined spectrophotometrically using solvent specific extinction coefficients (Rodriguez-Amaya and Kimura, 2004) and dried down under nitrogen gas. The dried carotenoid residues was reconstituted with cell culture medium containing 1% dimethyl sulfoxide (DMSO) at concentrations between 0-20 µM and bile salts diluted at final concentration range of between 0 and 10 mM and added to the cells. The control cells contained DMEM medium only. At the end of the incubation period, the medium was discarded and the cells carefully washed with DPBS. The medium was replaced with 100 µL MTT working solution and the plate incubated in the dark for 45 minutes. Then, the MTT solution was discarded, 1 mL DMSO added into each well and the plate shaken for 10 minutes at 35 rpm using a horizontal mini orbital shaker (Stuart, UK) to dissolve the formed formazan crystals. In triplicate, 200 µL of the solubilised formazan solution was transferred into a 96-well plate and cell viability measured at 570 nm and background subtraction using 620 nm reading via plate reader (Tecan, UK). Cell viability was expressed as percent using the formula:

$$\text{Cell viability (\%)} = \frac{Asample - Ablank}{Acontrol - Ablank} \times 100 \qquad \text{Equation 2-8}$$

Where;

$A_{control}$ is the absorbance of the cells incubated with the DMEM only;

$A_{sample}$ is the absorbance of the cells incubated with the sample;

$A_{blank}$ is the absorbance of the cell-free wells containing just DMSO

### 2.4.6 Cell differentiation measurement – Alkaline phosphatase activity assay

Upon reaching confluence, Caco-2 cells spontaneously differentiate into polarised enterocytes expressing high levels of brush border enzymes typical of small intestinal epithelial cells such as peptidases, disaccharides and alkaline phosphatase. Among these enzymes are the alkaline phosphatase (ALP) enzymes which gradually increase after cell confluence reaching a plateau

after 2-3 weeks of culture and can therefore be used as reliable markers to evaluate differentiation of Caco-2 cells (Ferruzza et al., 2012). The principle of the assay is that, ALP in alkaline environment, catalyses the hydrolysis of p-Nitrophenyl phosphate (p-NPP) to inorganic phosphate and p-Nitrophenol (p-NP), a yellow soluble end-product whose colour intensity is proportional to the enzyme activity. In this experiment, ALP assay in situ protocol was used following the procedure described by Ferruzza et al. (2012) with some modifications. Briefly, working solution made up of 100 mM diethanolamine, 150 mM NaCl and 2 mM $MgCl_2$ was adjusted to pH 9.5 with 5 M HCl. To this was added 2.5 mg/mL p-NPP. The ALP collection buffer was made with 10 mM Tris-HCl, 150 mM NaCl and adjusted to pH 9.5. Before the experiment, the working solutions were placed in a water bath and pre-warmed at 37°C. The cell monolayer was then washed twice with DPBS (in both AP and BL compartments). The reaction buffer (1 mL) was then added to both compartments and incubated for 30 mins. A volume of 150 µL was collected from the AP compartment after 30 min-incubation and transferred to a 96-well plate placed on ice. The reaction was then stopped with 50 µL NaOH. Absorbance was then read at 405 nm using the TECAN reader and results converted to concentration (nmol) after blank subtraction with reference to the standard curve.

### 2.4.7 Tissue system – Ussing chamber analysis

To investigate the absorption kinetics of carotenoids by *ex vivo* small intestinal tissue, the Ussing chamber methodology, consisting of two half chambers with fresh intestinal tissue mounted as a semi-permeable membrane between the two halves. The method as described previously (Mackie et al., 2019), involved the collection of the whole length of the digestive tract from 6-8 weeks old C57BL/6 mice. These animals have advantages that include similar genetic make-up to humans and their relative small body size that facilitates easy handling and large scale/high throughput studies (Vandamme, 2014). The collected tract was then flushed through with ice-cold 10 mmol/L glucose solution. To avoid the lymphoid follicles, the most distal part (1 cm) was discarded and a section of duodenum taken and cut along the mesenteric attachment. The cut section was washed with 10 mmol/L glucose and the serosal and muscularis layers stripped off using fine forceps. The tissue segment was then mounted on a slider (P2404, Physiologic instruments, San Diego, USA), which was later inserted into an Ussing chamber system (EM-CSYS-4 with low volume P2400 chamber), separating the chamber into the apical and the basolateral compartments leaving the exposed tissue area of 0.25$cm^2$. The apical compartment was filled with 1 mL Ringer's solution containing 10 mmol/L mannitol to maintain osmotic balance while the basolateral compartment was filled

with a similar volume of Ringer's solution containing 10 mmol/L glucose. The buffers in both compartments were continuously bubbled with carbogen (95% $O_2$ and 5% $CO_2$) while maintaining the samples at 37°C.

### 2.4.8 Tissue viability measurements

After stripping and mounting, mouse tissue was then equilibrated for 30 minutes to achieve steady state conditions in transepithelial potential difference (PD). This open-circuit transepithelial potential difference was monitored on a continuous basis using a DVC-1000 multichannel voltage clamp unit (World Precision Instruments, New Haven, USA) with Ag-AgCl electrodes and 150 mmol/L NaCl salt bridges. At 20-min intervals, a short circuit current (Isc) was applied to zero the PD with recordings collected using Spike2 v8.08 software (Cambridge Electronic Design, Cambridge, UK) and used to calculate transmucosal resistance using Ohm's law. The calculated resistance serves as a measure of tissue integrity throughout the experimental period. During the initial equilibration period, transepithelial resistance was assessed by measuring voltage changes in response to micro-amp pulses lasting a few seconds, applied every 2 minutes. As a control measure, tissue samples that recorded a PD < 1 mV were excluded from further analysis. As a marker of cellular integrity whose permeability is proportional to the damage levels of the tight junctions, CH dilithium Lucifer Yellow (LY) (Sigma Aldrich, UK) prepared at a final concentration of 0.1 mg/mL in HBSS was added to the apical section of the Ussing chambers at the beginning of the experiment and sampled from the basolateral section at the end of the experiment. The activity of LY was then measured using the TECAN reader with permeability flux estimated using the following equation:

$$\text{Percent permeability (\%)} = \frac{Absorbance\ basolateral}{Absorbance\ apical} \text{ x } 100 \qquad \text{Equation 2-9}$$

### 2.4.9 Tissue permeability studies

When electrical parameters stabilized, buffers from both compartments were removed and at time 0, Ringer's solution in the apical compartment replaced with 500 µL Ringer's + 500 µL digested emulsion whose final volume contained 10 mmol/L mannitol. Similarly, the buffer in the basolateral compartment was replaced with 500 µL Ringer's + 500 µL simulated intestinal salt solution with the final volume containing 10 mmol/L glucose. The simulated intestinal fluid was prepared according to the standardised INFOGEST static digestion protocol (Brodkorb et al., 2019) to achieve 100 U/mL of pancreatin in the final digestion mixture. The working solution of 1X Ringer's containing 10 mmol/L mannitol that was used in the apical compartment of the Ussing chamber was prepared from 5X concentration of both Ringer' and

mannitol solutions. During the course of digestion, a 200 µL aliquot was collected from both apical and basolateral compartments at 0, 30, 60, 90 and 120 min and the same volume replaced with fresh and previously warmed Ringer's solution containing 10 mmol/L mannitol and glucose respectively. The collected samples were analysed for carotenoids following procedures described in section 2.5.6. Upon completion of the experiment, it is expected that the tissue used in the experiment loses viability. Therefore, to check for transepithelial conductance, 10 µM forskolin was added to the compartments as a way of confirming reduced or non-response of the tissue after the experiment. Forskolin, a diterpene, has been reported to elicit a robust Glucagon-like peptide-1 (GLP-1) secretory responses in viable tissues (Brighton et al., 2015)

### 2.4.10 Carotenoid uptake and transport calculations

Percentage cellular uptake was calculated as a ratio of carotenoids accumulated in the Caco-2 cells against what was initially used in the experiment given by the following formula;

$$\text{Cellular uptake (\%)} = \frac{Carotenoid\ conc.\ in\ cells}{Initial\ carotenoid\ conc.(digesta)} \text{ x } 100 \qquad \text{Equation 2-10}$$

Cellular transport was calculated as a ratio of carotenoids accumulated in the cells + carotenoids secreted to the basolateral section against what was initially present given by the following formula;

$$\text{Cellular transport (\%)} = \frac{Conc.of\ carotenoids\ in\ cell+basolateral}{Initial\ carotenoid\ conc.(digesta)} \text{ x } 100 \qquad \text{Equation 2-11}$$

### 2.4.11 Extraction and quantification of carotenoids from food and test samples

Finely ground sample (0.3 ± 0.05 g) was weighed and placed into a labelled medium test tube with a screw cap (15 mL tube). To this tube, 3 mL ethanol containing 0.1%, w/v BHT was added and mixed by vortexing for 30 seconds. The tubes were then immediately covered and placed in a water bath previously heated at 85°C for 5 minutes. After this incubation time, tubes were removed from the water bath and 250 µL of 44%, w/v Potassium hydroxide (KOH) prepared as 80 g in 100 mL water, added and mixed by vortexing for 30 seconds. After this, the tube was flushed with nitrogen gas before closing them. Tubes were then placed in the 85°C water bath for another 5 min and removed when this time elapsed and mixed by vortex for 30 seconds. Tubes were then placed in the heated water bath for the last 5 min after which period they were removed, vortexed for 30 seconds and immediately placed on ice. After this, 1.5 mL of cold milli Q water was added. This was followed by the addition of 100 µL purified β-apo-

8’-carotenal (at approx. absorbance of 0.6) and mixed by vortexing for 30 seconds. To the saponified sample, 1.5 mL hexane was added and mixed for 30 seconds followed by centrifugation at 2400 g at 4°C for 5 minutes. Using a glass Pasteur pipette, the top hexane layer containing the partitioned carotenoids was carefully removed and placed into a new test tube. Extraction was repeated twice or until colour disappeared and the extract placed in the same tube. The combined extract was centrifuged at 2400 x *g* at 4°C for 5 minutes so that possible contaminants would settle at the bottom of the test tube. From the combined extract, a 2.5 mL aliquot of the hexane phase was collected and placed in a new 15 mL test tube and placed in heating block previous set at 30°C and the hexane extract completely dried under a steady flow of nitrogen gas. The dried extract was then reconstituted in 500 µL methanol:dichloroethane (50:50, v/v) vortexed for 30 seconds or until all visible particles adhering to the glass were dissolved. The dissolved sample was then filtered on a 0.22 µm Polytetrafluoroethylene (PTFE) filter and placed in 2 mL HPLC vial before inserting it in the HPLC auto-sampler previously set at 4°C and 20 µL sample injected into the system for analysis.

### 2.4.12 Extraction from cells, apical and basolateral solutions

Extraction of carotenoids from scrapped cells, apical and basolateral solutions was done following the procedure described by Reboul et al. (2005) with modifications. To the glass tube containing scraped cells or sample solution, 3 mL ethanol containing 0.1% (v/v) Butylated hydroxytoulene (BHT) was added to denature the cells and 100 µL of β-apo-8’-carotenal (abs = 0.204) as an internal standard added before closing the glass tube with a lid, mix and vortexing for 30 seconds. Hexane (6 mL) was then added to the glass tube, the lid closed and vortexed for 30 seconds. This was followed by sample centrifugation for 5 minutes at 4°C in the centrifuge (Eppendorf, UK) at 2,500 *g*. The hexane (upper layer) containing partitioned carotenoids was extracted with a clean glass Pasteur pipette. Exhaustive extraction was completed by repeating the extraction with 6 mL of hexane twice before combining the extracts and drying under a steady flow of nitrogen gas. The dried extract was then stored at -80°C until LCMS analysis.

## 2.5 Chromatographic analysis of carotenoids

### 2.5.1 Principle of HPLC analysis

HPLC employing detection methods such as diode array detector (DAD) is the method of choice for the quantitative determination of carotenoids in food samples (Preedy, 2012). While both the normal and reverse-phase system can be used in the separation and quantification of carotenoids, it is the reverse-phase (RP-HPLC) system that is generally preferred because of its advantages related to greater column stability and better reproducibility of the retention times in comparison to the normal phase system. The separation of carotenoids using the RP-HPLC is governed by polarity, hydrophobicity and ionic strength of molecules being separated (Jandera, 2012). It has been reported previously that the best separation of carotenoids has been achieved with the use of C30 chemically bonded stationary phase that can facilitate the quantification and separation of structurally similar and geometric isomers (Kopec et al., 2012). For consistent separation and prevention of carotenoids from crystallization, column temperatures are kept at above 25°C (Scott and Hart, 1993).

### 2.5.2 HPLC-DAD analysis of carotenoids

The analysis of carotenoids was based on a method by Muzhingi et al. (2008) with modifications. The identification and quantification of carotenoids was performed on the UFLCXR system (Shimadzu, Kyoto, Japan) with a binary pump and a PDA detector (SPD-20A), a solvent delivery module (LC-2AD), a degasser (DGU-20A3/A5), an auto-sampler unit (SIL-20A). The DAD was set at wavelengths 290, 325 and 450 for the simultaneous detection of tocopherols, retinoids and carotenoids respectively. Chromatographic separation of carotenoids was performed on a YMC C30 carotenoid column (3.9 mm x 250 mm, 3µm particle size, Waters Corporation, Ireland) at a flow rate of 0.9 mL/min. The column temperature was set at 30°C and injection volume of 20 µL. A gradient system consisting of 95% methanol containing 10 mM ammonium acetate as a solvent stabiliser as mobile phase (A) and 100% MTBE as mobile phase (B) was employed for this analysis. The elution program was as follows: Initial condition of 85% (A) with an increase to 37% (B) from 0-25 min, increases to 63% solvent (B) between 25-35 min, solvent (B) remained at 63% for 1 minute before returning to the initial conditions for the next 4 minutes. Identification of carotenoids was done by comparing their retention times to those obtained from authentic standards or UV-visible

spectrum and spiking of samples with authentic standards. For carotenoids without standards, identification was based on similar characteristics with published literature. The limit of detection (LOD) was determined from the amount of the analyte required to give a signal to noise ratio of 3 and while the limit of quantification (LOQ) was defined as the amount needed to give a signal to noise ratio of 10. The LOD (0.44 ng/µL) and LOQ (1.33 ng/µL) were determined using parameters of the analytical curve (Petry and Mercadante, 2018) as shown in Figure 2-2. Processing of data was done using Lab Solutions program (Shimadzu).

### 2.5.3 HPLC method optimisation

Prior to the analysis of test samples in this experiment, optimisation of the method was done using control food samples and the method was effectively used on chickpea from ICRISAT and tomato samples from Lancaster University, through a collaborative study that resulted in the establishment of LOD and LOQ values. Reproducibility was tested by using obtained peak areas of β-apo-8'-carotenal standard in the quantification of carotenoids Chickpea 'stay green' samples. The internal standard was prepared on the first day of the experiment and aliquots containing similar volumes were dried under nitrogen gas and stored at -80°C to be analysed on 4 different days. The mean peak areas were obtained from triplicate measurements and inter and intra-day coefficient of variation (CV) calculated as shown in Table 2-3

Table 2-3: Results of the inter and intra-day peak areas of β-apo-8'-carotenal standard

| Day | PK Area 1 | PK Area 2 | PK Area 3 | Mean | SD | CV (%) |
|---|---|---|---|---|---|---|
| 1 | 551,827 | 625,792 | 616,287 | 597,969 | 40,241 | 7 |
| 2 | 617,496 | 555,131 | 606,622 | 593,083 | 33,314 | 6 |
| 3 | 586,684 | 590,765 | 556,647 | 578,032 | 18,632 | 3 |
| 4 | 547,099 | 606,029 | 591,030 | 581,386 | 30,626 | 5 |
| 5 | 571,817 | 592,439 | 601,761 | 588,672 | 15,323 | 3 |
| **Average** | **574,985** | **594,031** | **594,469** | **587,828** | **27,627** | **5** |

The intra-day coefficient of variation on day 1 and 2 were above 5 and either 5 or below in the subsequent days presented as mean, SD and CV (%). Granted that the average CV was less than 5%, demonstrates excellent reproducibility of the experimental results and the optimised method was subsequently used in the analysis of the newly developed chickpea 'stay green' varieties leading to a peer-reviewed publication entitled "*Functional Dissection of the Chickpea (Cicer arietinum L.) Stay-Green Phenotype Associated with Molecular Variation at*

*an Ortholog of Mendel's I Gene for Cotyledon Colour: Implications for Crop Production and Carotenoid Biofortification*" by Sivasakthi et al. (2019).

Although the present experiment did not involve development of a new method, it was imperative to assess the LOD and the LOQ as the concentrations obtained in the analysis of carotenoids are low and require highly sensitive instruments. As indicated in Table 2-3, the precision of the HPLC instrument was evaluated by measuring the inter- and intra-day values of the peak areas using a known concentration of the internal standard. As the name suggests, the inter-day repeatability was assessed on the basis of 3 determinations conducted on the same day while the intra-day analysis was done over a period of 10 days to avoid losing the sample through degradation. Accuracy of the method was measured by spiking samples with known concentrations of *all-trans-β*-carotene, β-cryptoxanthin and lutein external standards while percentage recovery was estimated based on the area under the curve of the of the internal standard with no extraction against area of the internal standard with extraction.

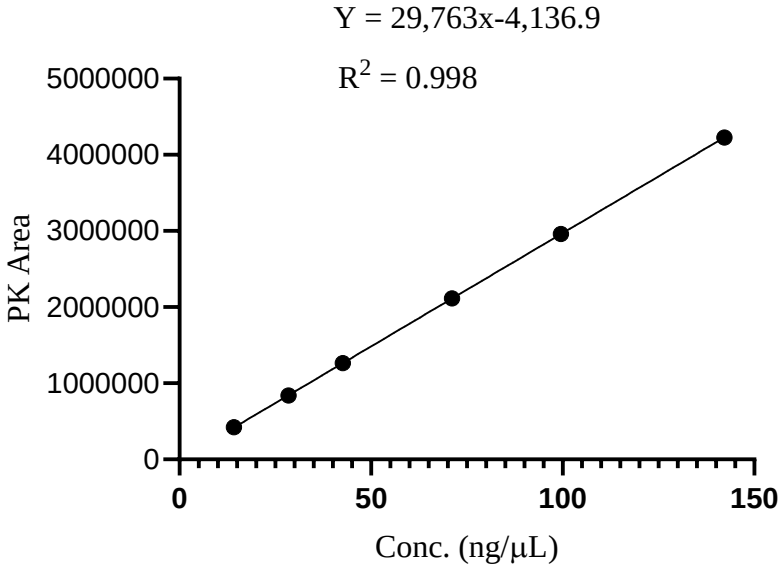



Figure 2-2: Calibration curve used for the estimation of LOD and LOQ during HPLC method optimisation. All data presented in the figure are means ± SD (n = 3).

Calculated from the following equations:

| | | |
|---|---|---|
| LOD = 3.3 * SD of intercept/slope | 0.441438726 ng/µL | Equation 2-12 |
| LOQ = 10 * SD of intercept/slope | 1.337693109 ng/µL | Equation 2-13 |

### 2.5.4 Principle of LCMS-MS analysis

The LCMS-MS work on the principle of separating individual components in a mixture using liquid chromatography followed by ionization and separation of the ions on the basis of their mass to charge ratio. The separated ions are then directed to a photo or electron multiplier tube detector where identification and quantification of ions is done. Information obtained from the detector differentiates compounds with diverse molecular masses. The LCMS-MS has a comparative advantage over the HPLC as it provides better sensitivity and specificity.

### 2.5.5 LCMS-MS analysis of carotenoids

Previously extracted and dried carotenoids were reconstituted in methanol:MTBE (60:40, v/v) mobile phase. For every batch of samples run, 1 µL sample volume of different concentrations of each carotenoid was injected into the ACQUITY UPLC I-Class PLUS (Waters, Wilmslow, UK) LCMS-MS system interfaced with the Xevo TQ-XS tandem mass spectrometer for mass detection. Data acquisition and processing was done using MassLynx software version 4.1. Optimised MS conditions were as follows: Electrospray Ionisation (ESI) performed in the positive ionisation mode, Capillary voltage 1.73 Kv, cone voltage: 41.27 V, source temperature: 150°C, desolvation temperature: 300°C, cone gas flow: 1 L/hr, desolvation gas flow: 798L/hr, collision gas flow on. For $ESI^+$, high purity nitrogen was used as the nebulization and desolvation gas at flow rates of 50 and 300 L/hr respectively. Optimal $ESI^+$-MS in the selected ion mode (SIM) and full scan (*m/z* 200-650) modes, as well as $ESI^+$-MS-MS in the multiple reaction monitoring mode (MRM) mode, were assessed and compared for the quantification of carotenoids. Chromatographic separation of carotenoids was achieved using the ACQUITY UPLC BEH 130Å C18 column (1.7 µm, 2.1 x 150 mm, Waters, Wilmslow, UK) at a flow rate of 0.360 µL/min and column temperature set at 30°C following the protocol by Li et al. (2005) with modifications. Reverse phase elution of carotenoids was performed using a gradient method with mobile phases of 95% methanol containing a mixture of 0.01M Ammonium acetate and 0.1% v/v formic acid (A) and methyl tert-butyl ether (MTBE) (B) with a column temperature set at 30°C. The following was the gradient elution programme: An isocratic mixture of methanol (87%) and MTBE (13%) was maintained from time 0 to 12 min, followed by a linear gradient until 25 min at which time the mobile phase composition was methanol (63%) and MTBE (37%). From 25-35 min, MTBE increased to 63% and was maintained for 1 min before returning to initial concentrations. After each sample run, the LCMS system was flushed with 50% methanol for lines that used 95% methanol containing ammonium acetate while lines using a stronger mobile phase (MTBE) were flushed

with 100% methanol to remove strongly retaining residuals. Carotenoids were identified according to their relative retention time and accurate mass (*m/z* 568.43 for lutein, *m/z* 536.44 for β-carotene and *m/z* 552 for β-cryptoxanthin for precursor ions and corresponding product ions at *m/z* 463 and 551 for lutein, *m/z* 460 for β-cryptoxanthin and *m/z* 536 for β-carotene). Calibration curves of pure carotenoid standards were constructed for carotenoid quantification from the original standard mixture (10 ng/µL) and serially diluted ranging from 50 to 1600 pg/µL.

### 2.5.6 Quantification of carotenoids

Quantification of carotenoids was achieved using standard curves prepared from individual external standards at six concentrations with correlation coefficient of greater than 0.9. The concentration of carotenoids was expressed as microgram per gram (µg/g) on a dry weight basis using the following formula;

**Extraction efficiency calculations**:

$$EE = \frac{AUC\ of\ internal\ standard\ with\ no\ extraction}{AUC\ of\ internal\ standard\ with\ extraction} \qquad \text{Equation 2-14}$$

Where;

EE = extraction efficiency

AUC = area under the curve (Detector response)

**Carotenoid concentration calculations**:

$$C = \frac{AUC \pm b}{m} * \frac{Reconstitution\ volume\ (\mu L)}{Injection\ volume\ (\mu L)} * \frac{Hexane\ vol\ after\ extraction\ (mL)}{Hexane\ aliquot\ evaporated\ (mL)} * W\ (g) * 1000$$

Equation 2-15

$C_{Adjusted} = C * EE$

Where;

C = carotenoid concentration (µg/g), Dry weight

AUC = area under the curve

b = intercept of calibration curve for respective standard

m = slope of the calibration curve for respective standard (AUC units per ng injected)

W = weight sample extracted (g)

$C_{Adjusted}$ = carotenoid concentration (µg/g), adjusted after losses during extraction.

# CHAPTER 3

# Effects of processing on the carotenoid content in butternut squash, biofortified orange-fleshed sweet potato and orange maize varieties

## 3.1 Summary

There is sufficient evidence on the effects of processing and post-harvest handling procedures on carotenoid content in foods rich in provitamin A carotenoids. However, information on the retention and stability of dietary carotenoids following different processing methods remains scanty and often conflicting, particularly when foods are prepared as composite meals and using traditional methods. In this study, different varieties of biofortified maize and orange-fleshed sweet potato (OFSP) and ordinary butternut squash (BNSQ) were prepared as individual as well as composite meals. Composite meals consisted of either *Nshima* from maize meal or OFSP with stew prepared under traditional methods. The different processing and meal preparation methods were then investigated for carotenoid content, retention and stability. All the processing methods led to a decrease in the content of all-*trans*-β-carotene, the compound with the highest provitamin A activity in all the food samples, by about 11-26% and 7-21% in biofortified maize and OFSP genotypes respectively. The decrease was more pronounced in BNSQ were losses in all-*trans*-β-carotene of up to 46.6% were recorded. Boiling and composite meal preparation improved provitamin A retention by over 100% in OFSP and maize meal respectively. Carotenoid type and genotypic effect significantly affected both retention and stability in biofortified food samples. All-*trans*-β-carotene in biofortified maize samples degraded by about 50% when stored at -20°C for 15 days. The results support the notion that optimised food processing and preparation methods can be used to derive maximum benefits in consuming biofortified food products.

*Keywords*: Carotenoid, composite meal, all-trans-β-carotene, processing.

## 3.2 Introduction

Maize (*Zea mays*) and sweet potato (*Ipomoea batatas*), produced as cereal and root crops respectively, are consumed as staple foods by over a billion people worldwide using different processing methods (Chitchumroonchokchai et al., 2017). Without doubt, these foods have succeeded in meeting the energy needs of poor populations in the developing countries (Bouis et al., 2011). In addition to this is butternut squash (*Cucurbita moschata* Duchesne), a food vegetable not only high in iron, zinc and vitamin A nutrients but also one that is highly consumed, is economical and available through most parts of the year in regions where it is consumed (Evangelina et al., 2001). These foods constitute excellent sources of provitamin A carotenoids in the human diet and therefore, their consumption is being promoted in sub-Saharan African countries as a food-based strategy to complement existing efforts in reducing the occurrence of VAD in the region (Burri et al., 2011; Gannon et al., 2014; Bouis and Saltzman, 2017; Chitchumroonchokchai et al., 2017; Awuni et al., 2018). The assumption is that sustained consumption of these nutrient dense staple crops can substantially reduce micronutrient deficiencies through daily adequacy of dietary intakes among individuals throughout the life cycle (Bouis et al., 2011). VAD is a disease of public health importance that affects over 190 million children under the age of 5 especially in Africa and Southeast Asia (Blomme et al., 2020). As humans cannot synthesise vitamin A *de novo*, its acquisition is through dietary intake of foods of animal origin in the form of preformed vitamin A (retinol) and/or plant origin in the form of provitamin A carotenoids (Tang and Life, 2013; Blomme et al., 2020). While consumption of nutrient enhanced foods holds the promise for sustainable and cost-effective food-based solutions to combat micronutrient deficiencies (Gupta et al., 2015a), of major concern however is the fact that the carotenoids in these staple crops are highly susceptible to postharvest degradation when exposed to heat, oxygen and light owing to their unsaturated structures (De Moura et al., 2015). Several studies have demonstrated the effect of different types of processing on the content and stability of carotenoids in different food matrices and plant genotypes. For instance, comparison of three processing methods (cooking in boiling water for 20 minutes, frying at 170°C and steam cooking for 10 minutes) in nine sweet potato cultivars revealed that frying led to a greater loss of total carotenoid and β-carotene than any other processing method used (Kim et al., 2015a). The type of processing employed has equally been shown to have a critical role in the retention of carotenoids in food samples. Blanching for 20 minutes followed by solar drying at temperatures ranging from 45 to 63°C for 10 hours, led to *trans*-β-carotene retention of 91.1% in orange pulp sweet potato

chips (Bengtsson et al., 2008; Júnior et al., 2020). Interestingly, different studies have produced varying results on the effects of processing on the stability of carotenoids. The stability and content of carotenoids were found to be different even after applying the same processing method to the same sample (Reboul et al., 2006; Koh and Loh, 2018). Apart from the ordinary physical processing methods, butternut squash, maize and sweet potato are processed in a variety of ways with the common method being that of cooking by boiling, frying and baking. While a number of studies have reported the effect of processing on carotenoid content, information on retention and stability of carotenoids following different processing methods remains scanty and often conflicting. This is especially true when foods are further prepared as composite meals, where interactions between ingredients may occur. Currently, very little if anything is known about the content, retention and stability of carotenoids in composite meals. In this chapter, individual foods and their composite meals with high provitamin A carotenoid content were specially selected and subjected to different processing and preparation methods in order to investigate their content, retention and stability. Therefore, the aim of this chapter was to investigate the content, retention and stability of carotenoids following different processing and meal preparation methods in butternut squash, biofortified maize meal and orange-fleshed sweet potato varieties. Results of this investigation could potentially be used to improve and optimise cooking methods on one hand and help potato tuber and maize breeding institutions set appropriate targets to counter projected losses emanating from processing methods on the other hand.

## 3.3 Methodology

The primary focus of this investigation was to explore the relationship between food processing methods and carotenoid content, retention and stability in both individual and composite meals. In this regard, different genotypes of biofortified maize grain were processed into flour using methods that typically involve turning the grain into maize flour to produce porridge or cooked dough called *Nshima*. Meals were prepared from *Nshima*, OFSP and Butternut squash (BNSQ), using traditional processing methods described in **section** 2.2.2. Carotenoids and vitamin E extracted from these samples were quantified using the HPLC-DAD coupled to the fluorescence detector following the method described by (Muzhingi et al., 2008; Rodrigues et al., 2016) as detailed in **section** 2.4.11. Separation and identification of carotenoids and vitamin E was achieved on a C30 column from which analytes eluted using a gradient method at a flow rate of 0.9 mL/min. This gradient method facilitated the separation of carotenoids from their

geometric isomers. All experiments were performed with three replicates. The statistical analysis of collected data, were applicable, was performed by either a t-test or Analysis of variance (ANOVA) using GraphPad prism software and post hoc test (Tukey's test). Equality of variances was verified by box plots while normality was checked using the distribution of normality plots. Significance was accepted at $P < 0.05$.

## 3.4 Results

### 3.4.1 Moisture content

Moisture content was calculated on the basis of dry matter with varying results in all the food samples. The dry matter content for maize ranged from 30.2 - 35.6% for cooked maize meals, 25.5 - 34.9% and 33.7 – 41.5% in raw and baked OFSP and BNSQ respectively.

### 3.4.2 Carotenoid composition in food samples

Following extraction and analysis, a typical HPLC chromatogram of major carotenoids in the analysed food samples was obtained as shown in Figure 3-1 revealing that on a C30 column, carotenoids were easily identified. Identification of these compounds was achieved by retention time and absorption spectra collected between 200 and 600 nm with those of authentic standards. From the method optimised in section **2.5.4**, lutein, zeaxanthin, β-cryptoxanthin, 9-*cis*-β-carotene, all-*trans*-β-carotene and 13-*cis*-β-carotene carotenoids where identified by peak retention times occurring at 14.8, 16.5, 26.2, 31.2, 33.7 and 35.0 mins respectively. Similarly, vitamin E was identified on the Fluorescence detector with an excitation wavelength set at 290 and emission wavelength set at 330 nm in the differently processed biofortified maize and OFSP samples.

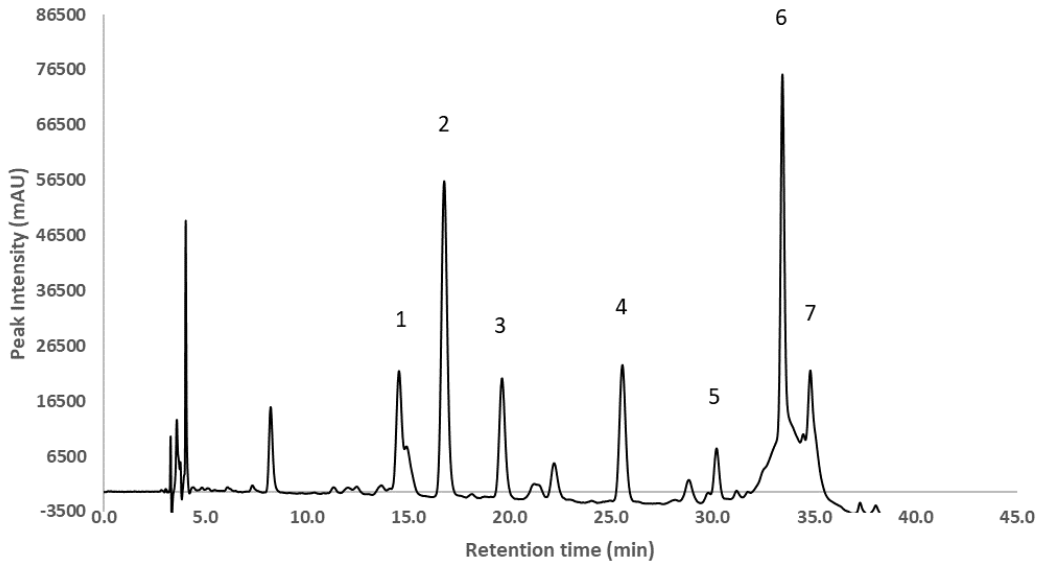



Figure 3-1: Representative chromatograms (abs 450 nm) of major carotenoids found in Maize, OFSP and BNSQ flour.

The chromatogram in Figure 3-1 has the following peaks identified: lutein (1), zeaxanthin (2), echinenone internal standard (3), β-cryptoxanthin (4), 13-*cis*-β-carotene (5), all-trans-β-carotene (6) and 9-*cis*-β-carotene. The peak after 9-*c*is-β-carotene could not be identified. Results of the baseline data and effect of processing on carotenoid content expressed in µg/g dry weight (DW) are presented in Table 3-1, Table 3-2, Table 3-3 and Figure 3-2. It is evident that the major carotenoids in the food samples were lutein, zeaxanthin, all-*trans*-β-carotene, β-cryptoxanthin, 13-*cis*-β-carotene and 9-*cis*-β-carotene. The variation in terms of carotenoid concentration depended on the food matrix. Provitamin A carotenoids, defined as **Pro-VITA** in our study, was computed as a summation of all-*trans*-β-carotene, half the value of β-cryptoxanthin (½ β-cryptoxanthin), half (½ 13-*cis*-β-carotene) and half (½ 9-*cis*-β-carotene).

### 3.4.3 Carotenoid content in biofortified maize

In the case of biofortified maize, the Pro-VITA carotenoids ranged from 2.23 to 5.10 µg/g DW among all the maize varieties (Table 3-1). In the unprocessed samples, the non-provitamin A carotenoids, lutein and zeaxanthin, were the major carotenoids in three out of the four genotypes of biofortified maize analysed. Among these genotypes, a combination of lutein and zeaxanthin accounted for 62.6% in GV662, 51.7% in GV 664 and 57.0% in GV 665 of the total carotenoids. All-*trans*-β-carotene was the main carotenoid in GV 672 accounting for 72.4% and 45.7% of Pro-VITA and total carotenoids respectively. There were very low concentrations of β-carotene isomers, 13-*cis* and 9-*cis*, while α-carotene, often found in provitamin A rich

foods, could not be detected. It is worth noting that GV 664 had all the main carotenoids, more or less, in similar concentrations at 2.57 ± 0.66 µg/g DW for lutein, 2.87 ± 0.68 µg/g DW for zeaxanthin, 2.60 ± 0.22 µg/g DW for β-cryptoxanthin and 2.98 ± 0.31 for all-*trans*-β-carotene. The vitamin E content was highest in GV 662 (4.66 ± 0.80 µg/g DW) and lowest in GV 672 (4.07 ± 0.03 µ/g DW).

There was a significant change in the content of vitamin E when ordinary maize was processed to *Nshima meal* ($P < 0.05$) (Figure 3-3) but not in the Pro-VITA carotenoid profile **(**Table 3-1**)**

All carotenoids were detected in the *Nshima* and *Nshima meal* save for 9-*cis*-β-carotene isomer which could not be detected in both preparation methods for GV 662 and one food preparation for GV 664 but was available in trace amounts in all the other maize genotypes. However, when the maize varieties were prepared as *Nshima*, processing caused a significant reduction in the contents of all the dietary carotenoids ($P < 0.01$) except for 13-*cis*-β-carotene isomer possibly due to the process of *trans*-*cis*-isomerisation and potential degradation of the carotenoids (Table 3-2).

Generally, there was variability in the carotenoid content in the maize genotypes, with GV 662 having higher contents of xanthophylls (lutein, zeaxanthin and β-cryptoxanthin). Neither α-carotene nor lycopene were found in detectable quantities in any of the food samples analysed.

### 3.4.4 Carotenoid content in biofortified OFSP

When biofortified OFSP genotypes were analysed, Pro-VITA ranged from 4.39 to 85.50 µg/g DW. In these varieties, *Kakamega* had significantly higher Pro-VITA (85.50 µg/g DW) compared to the other OFSP varieties ($P < 0.05$) Table 3-1. Like all the biofortified OFSP genotypes, all-*trans*-β-carotene was the major carotenoid with a concentration of 80.6 ± 3.10 µg/g DW in *Kakamega* genotype followed by 13-*cis*-β-carotene (4.36 ± 0.88 µg/g) and β-cryptoxanthin (4.28 ± 0.54 µg/g). Among the five (5) varieties of OFSP, *Kakamega* had the highest concentration of all-*trans*-β-carotene (80.6 ± 3.10 µg/g DW) followed by the three varieties in *Lunga*, *Yellow Santura* and *Chumfwa* whose concentration did not differ significantly from each other ($P = 0.521$). In comparison to the other biofortified OFSP varieties, *Chingovwa* had the lowest concentrations of all-*trans*-β-carotene at 4.4 ± 1.51 µg/g DW and therefore excluded from further investigations. Of the non-provitamin A carotenoids, only zeaxanthin was detected in minute quantities. This carotenoid accounted for 0.2% in *Kakamega*, 1.2% in *Lunga*, 0.9% in *Yellow Santura* and 0.8% in *Chumfwa* of the total

carotenoids in these food samples. Expectedly, there were very low concentrations of the β-carotene isomers, 13-*cis*-β-carotene and 9-*cis*-β-carotene due to minimal processing at baseline. Neither lutein, a non-provitamin A carotenoid nor α-carotene, a provitamin A carotenoid could be detected in the baseline samples.

The results on processing of biofortified OFSP carotenoid content are shown in Table 3-3**,** Figure 3-2 and Figure 3-3**.** When the biofortified OFSP varieties were subjected to boiling and baking processing methods, there was a reduction in the all-*trans*-β-carotene and zeaxanthin with increases in the β-cryptoxanthin and β-carotene isomers in all the varieties. In comparison to baking, boiling resulted in significant β-cryptoxanthin increases of up to 78.2% vs 25.7 % for *Kakamega*, 30.1% vs 9.1 for *lunga*, 48.8% vs 5.7% for *Yellow Santura* and 16.1% vs 13.8% for *Chumfwa* ($P < 0.05$). The increase after processing could be as a result of insufficient extraction capability by the chemical extraction method of the native material. In the β-carotene isomers, 13-*cis*-β-carotene specifically, it was baking in comparison to boiling that resulted in significant increases of up to 97.9% vs 17.2% in *Kakamega*, 39.0% vs 3.1% for *Lunga*, 29.5% vs 15.3% for *Yellow Santura* and 46.1% vs 19.6% for *Chumfwa* ($P < 0.05$). There was however no significant change in the levels of 9-*cis*-β-carotene between baked and boiled biofortified OFSP in all the varieties ($P > 0.05$). While processing resulted in increases of β-cryptoxanthin and in one of the β-carotene isomers, its effect caused some losses in the content of major provitamin A carotenoid, all-*trans*-β-carotene and the non-provitamin A carotenoid, zeaxanthin. In the case of all-*trans*-β-carotene, and in relation to boiling, baking resulted in losses of up to 21.5% vs 11.7% for *Kakamega*, 9.2% vs 3.7% for *Lunga*, 18.5% vs 15.1% for *Yellow Santura* and 11.8% vs 7.8% for *Chumfwa*. A similar pattern was observed for zeaxanthin except that for *Kakamega* variety, baking caused significant losses of 33.3% with no observable changes in the boiled product. The loss was more pronounced in *Chumfwa* which had 50% vs 35.9% when compared to the effects of boiling. This was followed by 39.7% vs 19.2% in *Yellow Santura* and 5.2% vs 2.1% in *Lunga* when compared to the effect of boiling. The concentrations of vitamin E ranged from 10.71 to 12.67 µg/g DW and processing did not in any way significantly affect content ($P > 0.05$).

Table 3-1: Baseline Carotenoid Content (µg/g DW) in biofortified maize and OFSP varieties

| **Variety/genotype** | **Lutein** | **Zeaxanthin** | ***β*-cryptoxanthin** | **All-*trans*-*β*-carotene** | **13-*cis*-*β*-carotene** | **9-*cis*-*β*-carotene** | **Pro-VITA** | **Vitamin E** |
|---|---|---|---|---|---|---|---|---|
| MAIZE VARIETIES | | | | | | | | |
| ***GV 662*** | 3.30 ± 0.95 | 4.85 ± 1.19 | 3.38 ± 0.29 | 2.51 ± 0.06 | 0.52 ± 0.11 | 0.82 ± 0.06 | 4.87 | 4.66 ± 0.80 |
| ***GV 664*** | 2.57 ± 0.66 | 2.87 ± 0.68 | 2.60 ± 0.22 | 2.98 ± 0.31 | 0.74 ± 0.09 | 0.86 ± 0.07 | 5.08 | 4.24 ± 0.59 |
| ***GV 665*** | 1.44 ± 0.57 | 1.52 ± 0.60 | 0.79 ± 0.02 | 1.59 ± 0.99 | 0.20 ± 0.02 | 0.28 ± 0.03 | 2.23 | 4.56 ± 1.06 |
| ***GV 672*** | 1.58 ± 0.28 | 1.39 ± 0.23 | 1.06 ± 0.09 | 3.69 ± 0.41 | 0.83 ± 0.17 | 0.92 ± 0.08 | 5.10 | 4.07 ± 0.03 |
| OFSP VARIETIES | | | | | | | | |
| *Kakamega* | ND | 0.15 ± 0.04 | 4.28 ± 0.54 | 80.6 ± 3.10 | 4.36 ± 0.88 | 1.16 ± 0.38 | 85.50 | 12.67 ± 0.53 |
| *Lunga* | ND | 0.95 ± 0.11 | 6.91 ± 0.59 | 67.5 ± 2.64 | 5.23 ± 0.10 | 0.25 ± 0.09 | 73.70 | 12.61 ± 0.89 |
| *Yellow Santura* | ND | 0.73 ± 0.12 | 4.94 ± 0.80 | 66.1 ± 5.29 | 8.64 ± 1.61 | 0.13 ± 0.04 | 72.96 | 11.08 ± 2.09 |
| *Chumfwa* | ND | 0.64 ± 0.08 | 5.09 ± 0.77 | 68.0 ± 4.43 | 4.69 ± 0.92 | 0.27 ± 0.08 | 73.03 | 10.71 ± 1.59 |
| *Chingovwa* | ND | 0.49 ± 0.10 | 1.71 ± 0.12 | 4.4 ± 1.51 | 2.03 ± 0.34 | 0.98 ± 0.44 | 4.39 | 11.47 ± 0.24 |

Data are means ± SD of three independent analyses (n=3) for each variety/genotype and preparation method; Pro-VITA = [all-*trans*-β-carotene + ½ β-cryptoxanthin + ½ 13-*cis*-β-carotene + ½ 9-*cis*-β-carotene]; ND = Not detectable.

Table 3-2: Carotenoid content in processed biofortified varieties of maize (µg/g DW)

| Variety/genotype | Lutein | Zeaxanthin | *β*-cryptoxanthin | All-*trans*-*β*-carotene | 13-*cis*-*β*-carotene | 9-*cis*-*β*-carotene |
|---|---|---|---|---|---|---|
| **MAIZE VARIETIES** | | | | | | |
| ***GV 662*** | | | | | | |
| Nshima | 2.21 ± 0.13 | 4.06 ± 0.14 | 3.07 ± 0.060 | 2.22 ± 0.04 | 0.61 ± 0.48 | *ND* |
| Nshima meal | 2.42 ± 0.06* | 4.38 ± 0.16* | 3.89 ± 0.26* | 2.56 ± 0.36 | 0.69 ± 0.34 | *ND* |
| ***GV 664*** | | | | | | |
| Nshima | 2.06 ± 0.04 | 2.16 ± 0.08 | 1.86 ± 0.08 | 2.19 ± 0.17 | 1.34 ± 0.18 | *ND* |
| Nshima meal | 2.12 ± 0.10 | 2.39 ± 0.11 | 2.08 ± 0.12 | 1.76 ± 0.09 | 0.99 ± 0.03 | 0.24 ± 0.06 |
| ***GV 665*** | | | | | | |
| Nshima | 1.14 ± 0.09 | 1.18 ± 0.19 | 0.64 ± 0.12 | 2.51 ± 0.83 | 0.90 ± 0.07 | 0.24 ± 0.04 |
| Nshima meal | 1.67 ± 0.32 | 0.83 ± 0.04 | 0.43 ± 0.04 | 2.67 ± 0.66 | 1.01 ± 0.10 | 0.29 ± 0.11 |
| ***GV 672*** | | | | | | |
| Nshima | 1.98 ± 0.24 | 0.86 ± 0.02 | 1.66 ± 0.17 | 3.65 ± 0.11 | 2.22 ± 0.27 | 1.42 ± 0.30 |
| Nshima meal | 2.14 ± 0.32 | 0.71 ± 0.11 | 2.03 ± 0.08 | 3.04 ± 0.05 | 2.65 ± 0.09 | 1.29 ± 0.05 |

Data are means ± SD of three independent analysis ($n$=3) for each variety/genotype and preparation method; ND = Not detectable; Asterisk (*) indicate significant difference within columns ($P < 0.05$) using a $t$-test.

Table 3-3: Carotenoid content and retention in processed biofortified OFSP varieties (µg/g DW)

| Variety/genotype | Lutein | Zeaxanthin | β-cryptoxanthin | All-*trans*-β-carotene | 13-*cis*-β-carotene | 9-*cis*-β-carotene | Retention (%) |
|---|---|---|---|---|---|---|---|
| **OFSP VARIETIES** | | | | | | | |
| ***Kakamega*** | | | | | | | |
| Raw | *ND* | 0.15 ± 0.04 | 4.28 ± 0.54 | 80.6 ± 3.10 | 4.36 ± 0.88 | 1.16 ± 0.38 | |
| Baked | *ND* | 0.10 ± 0.04 | 5.38 ± 0.19* | 63.3 ± 3.10* | 8.63 ± 1.68* | 2.32 ± 0.88 | 83.6 |
| Boiled | *ND* | ND | 7.63 ± 0.66* | 71.2 ± 1.23* | 5.11 ± 2.25 | 1.16 ± 0.80 | 91.4 |
| ***Lunga*** | | | | | | | |
| Raw | *ND* | 0.95 ± 0.11 | 4.91 ± 0.59 | 67.5 ± 2.64 | 5.23 ± 0.10 | 0.25 ± 0.09 | |
| Baked | *ND* | 0.90 ± 0.11 | 5.54 ± 0.59 | 61.3 ± 1.31* | 7.27 ± 0.98* | 0.52 ± 0.04 | 93.5 |
| Boiled | *ND* | 0.93 ± 0.07 | 6.99 ± 1.06 | 65.0 ± 1.12 | 5.39 ± 1.91 | 0.85 ± 0.69 | 102.6 |
| ***Yellow Santura*** | | | | | | | |
| Raw | *ND* | 0.73 ± 0.12 | 4.94 ± 0.80 | 66.1 ± 5.29 | 8.64 ± 0.61 | 0.13 ± 0.04 | |
| Baked | *ND* | 0.44 ± 0.06* | 5.22 ± 1.10 | 53.9 ± 5.33* | 11.19 ± 1.14* | 0.37 ± 0.19 | 112.8 |
| Boiled | *ND* | 0.59 ± 0.04* | 7.35 ± 0.08* | 56.1 ± 3.38* | 9.96 ± 1.15 | 0.15 ± 0.08 | 116.3 |
| ***Chumfwa*** | | | | | | | |
| Raw | *ND* | 0.64 ± 0.08 | 5.09 ± 0.77 | 68.0 ± 4.43 | 4.69 ± 0.92 | 0.27 ± 0.08 | |
| Baked | *ND* | 0.32 ± 0.05* | 5.79 ± 0.04 | 60.0 ± 2.22* | 6.85 ± 0.79* | 0.63 ± 0.08* | 91.2 |
| Boiled | *ND* | 0.41 ± 0.02* | 5.91 ± 0.19 | 62.7 ± 1.08* | 5.61 ± 0.96 | 0.30 ± 0.19 | 94.0 |

Data are means ± SD of triplicate analysis ($n$=3) for each variety/genotype and preparation method; ND = Not detectable; Asterisk (*) indicate significant difference within columns ($P < 0.05$) of the same sample using ANOVA.

### 3.4.5 Carotenoid content in ordinary OFSP and BNSQ

As part of baseline assessment, ordinary OFSP and BNSQ was procured from the local stores and analysed for carotenoid content. In both food samples, the carotenoid profile consisted of all-*trans*-β-carotene, β-cryptoxanthin and the *cis*-isomers of β-carotene. All-*trans*-β-carotene was the most abundant provitamin A carotenoid in the raw samples of both BNSQ and OFSP contributing 55.3% and 63.9% of the total provitamin A carotenoids respectively (Figure 3-2). This was followed by β-cryptoxanthin at 18.9% and 32.0% and lastly, the β-carotene isomer, 13-*cis* at 11.4% and 4.0% for BNSQ and OFSP respectively. In these ordinary BNSQ and OFSP varieties, baking was the only processing method utilized and this led to significant losses in the concentration of all-*trans*-β-carotene and β-cryptoxanthin in both food samples ($P < 0.05$). The reduction in content was significantly higher for β-cryptoxanthin at 69.6% in BNSQ against 8.1% in OFSP. Similarly, all-*trans*-β-carotene recorded losses of up to 46.6% in BNSQ against 22.3% in OFSP. Although the β-carotene isomers were detected in trace amounts in the raw samples, baking led to increased isomerisation of 13-*cis*-β-carotene and 9-*cis*-β-carotene of 4 to 5 times greater than the amount in the unprocessed samples in both food samples. The non-provitamin A could not be detected while vitamin E was not analysed in these samples.

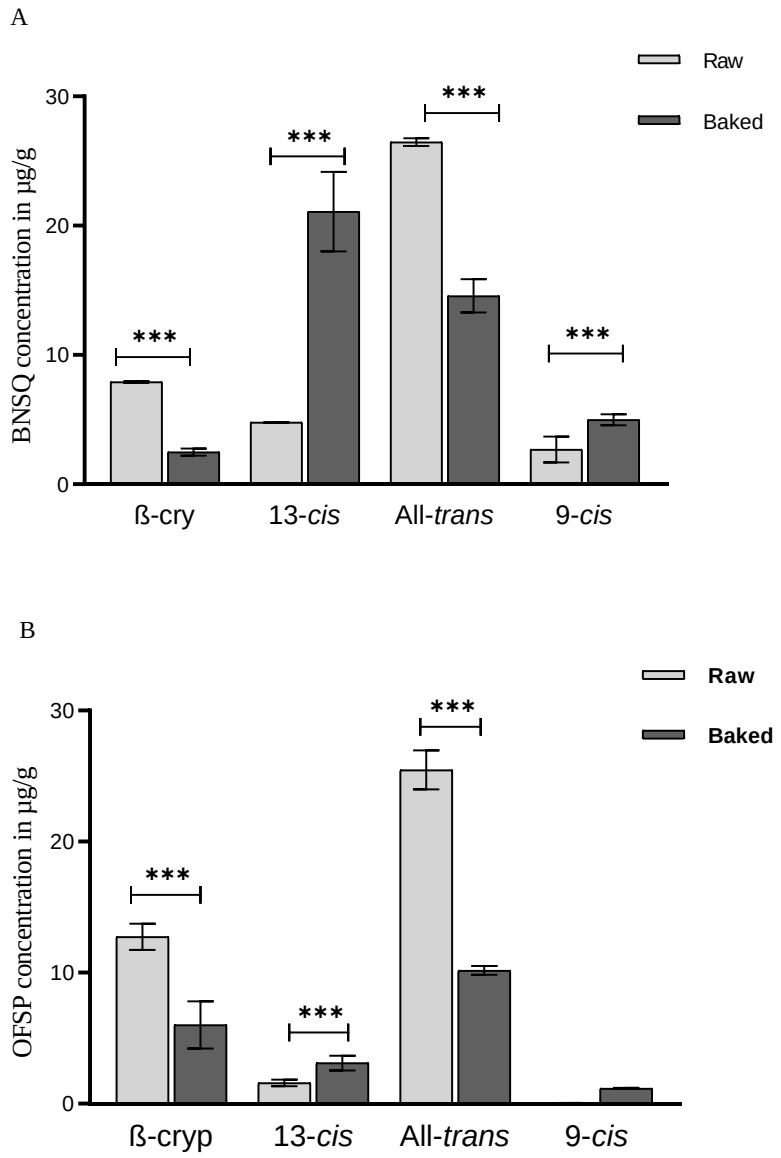



Figure 3-2: Effect of processing on the carotenoid content in ordinary BNSQ (A) and OFSP (B). Values presented as mean ± SD; ($n$ = 3). *** Highly significant.

### 3.4.6 Carotenoid retention in biofortified maize and OFSP

The percentage retention of carotenoids was obtained using the following equation:

$$\text{Percentage Retention (\%)} = \frac{carotenoid\ content\ per\ gram\ of\ processed\ food\ DW}{carotenoid\ conent\ per\ gram\ of\ raw\ food\ DW} \text{ X } 100 \quad \text{Equation 3-1}$$

Retention of carotenoids in processed samples is affected by losses due to degradation, *trans-cis*-isomerisation or gains by potential leakages as a result of food matrix disruption. In this experiment, both Pro-VITA carotenoids and vitamin E in the maize varieties were not significantly related to the type of processing ($P$ > 0.05) (Table 3-3). All maize genotypes

showed higher Pro-VITA carotenoids retention i.e. above 67.2%, GV 665 and GV 672 were the highest varieties with retention values of 152.5 – 158.5% and 118.1 – 123.5% respectively **(**Table 3-3**)**. This apparent retention increase was probably due to improved extractability after processing. The percentage of xanthophylls (lutein, zeaxanthin and β-cryptoxanthin) was highest in GV 662 representing 75% of total quantifiable carotenoids and lowest in variety GV 672 at 42% of the total carotenoids. This observation was particularly prominent when the composite *Nshima meal* prepared from ordinary *Nshima* resulted in the highest retention for almost all the carotenoids in every maize genotype. In biofortified OFSP, all varieties exhibited high retention values for Pro-VITA carotenoids with the lowest range being 83.6 – 91.4% for *Kakamega* and the highest being recorded for *Yellow Santura* (112.8 – 116.3 %) (Table 3-3). The retention for Pro-VITA varied from 83.6% in baked OFSP to 91.4% in boiled *Kakamega*, 92.9% to 100% in *Lunga*; 112.8% to 114.2% in *Yellow Santura*; 91.5% to 93.1% in *Chumfwa* for boiled and baked samples respectively. While *Kakagema* had high Pro-VITA carotenoid content, retention was significantly higher for both processing methods in *Yellow Santura* variety. For individual carotenoids, both processing methods showed greater retention for zeaxanthin with values ranging from 125.7% to 178.3% in *Kakamega*; 80.2% to 101.2 % in *Lunga*; 85.4% to 148.8% in *Yellow Santura* and 113.8% to 116.1% in *Chumfwa* for baked and boiled samples respectively (Figure 3-3**).**

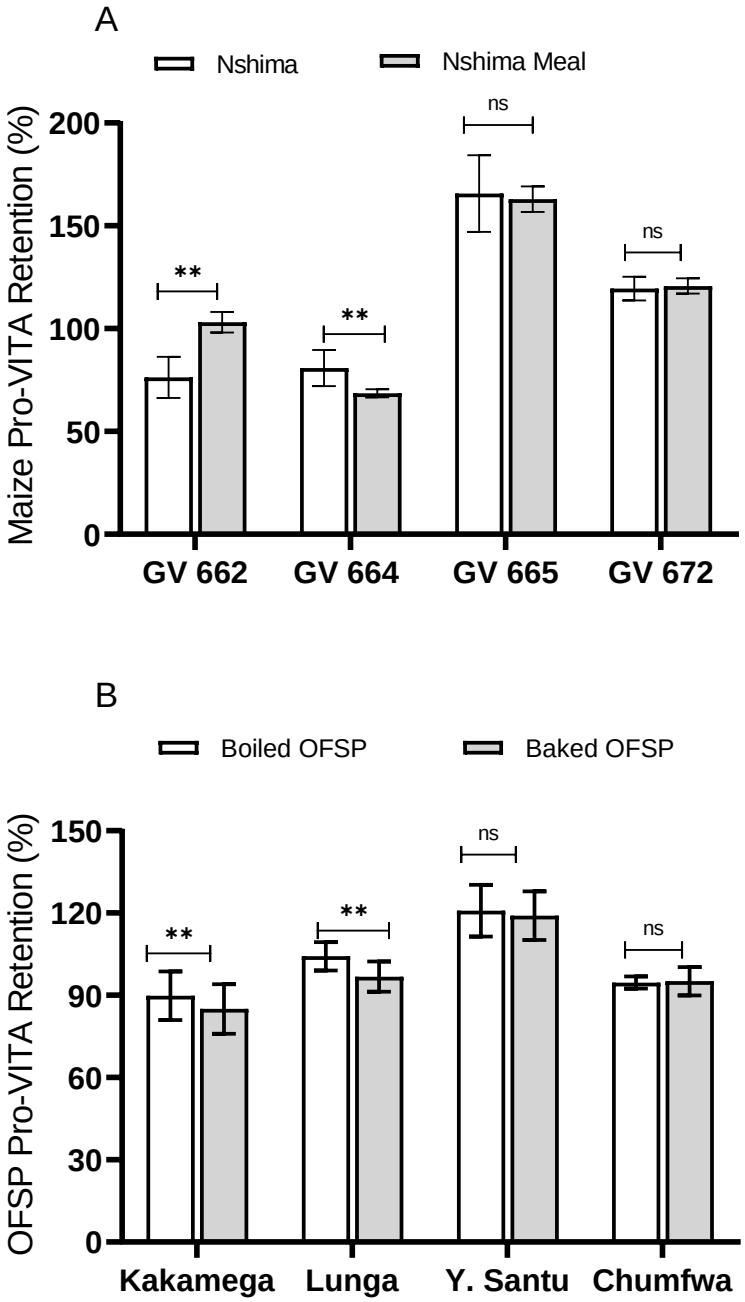



Figure 3-3: Effect of processing on Pro-VITA retention of carotenoids in processed biofortified maize and OFSP varieties. Values are means ± SD; ($n$ = 3); $P < 0.05$

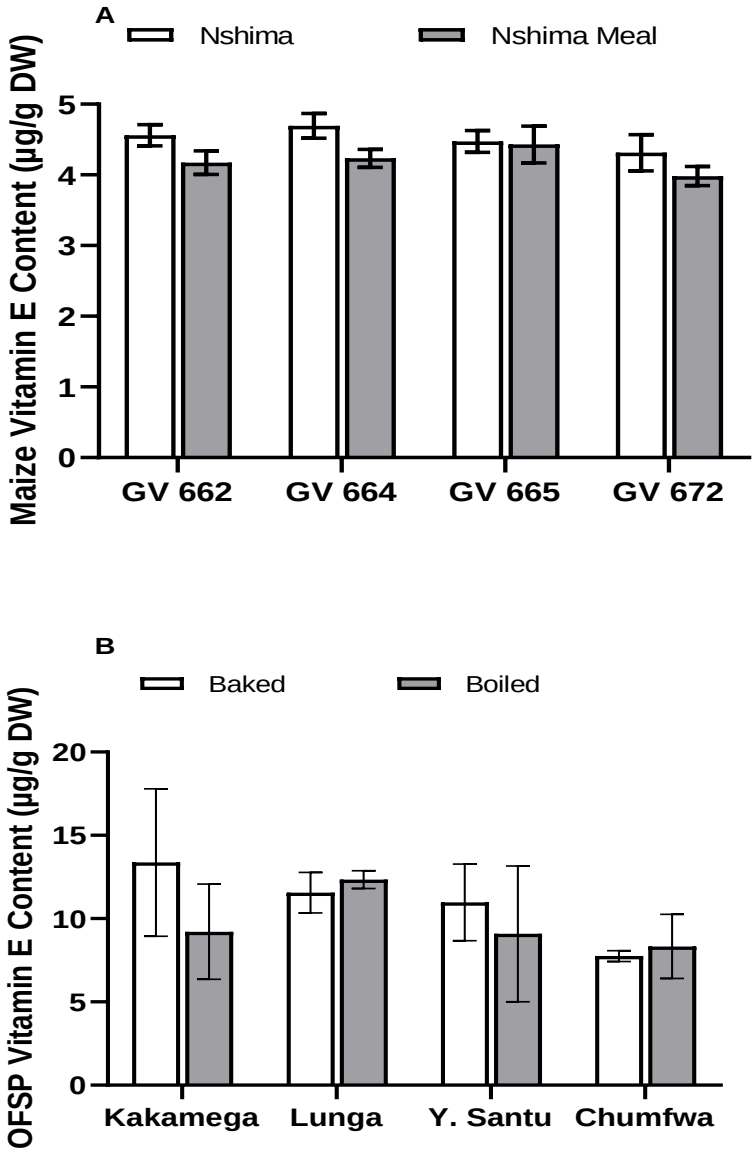



Figure 3-4: Vitamin E content in different biofortified (A) Maize and (B) OFSP varieties. Values are mean ± SD ($n$ = 3)

### 3.4.7 Carotenoid stability in biofortified maize varieties

One of the major factors that affect carotenoid stability and ultimate degradation is storage temperature. The stability of the carotenoids in the unprocessed biofortified maize meal samples were studied by assessing the effect of storage temperature on carotenoid stability over a period of 15 days (Figure 3-5). Carotenoids were extracted from the different maize genotypes with one set stored at -20°C for 15 days while the other was analysed immediately after the extraction procedure. Freezing at -20°C for 15 days led to a significant decrease in the hydrocarbon carotenoids (all-*trans*-β-carotene and its associated isomers) ($P < 0.05$) with percentage reductions of 46% in GV 672; 50.1% in GV 664; 53.2% in GV 665 and 54.6% in

GV 662. By contrast, the oxygenated carotenoids (lutein, zeaxanthin and β-cryptoxanthin demonstrated relative stability after 15 days storage at -20°C in all the genotypes.

In general, susceptibility of carotenoids to the effects of freezing at -20°C was significantly affected by the type of carotenoids in all the biofortified maize genotypes analysed ($P < 0.05$).

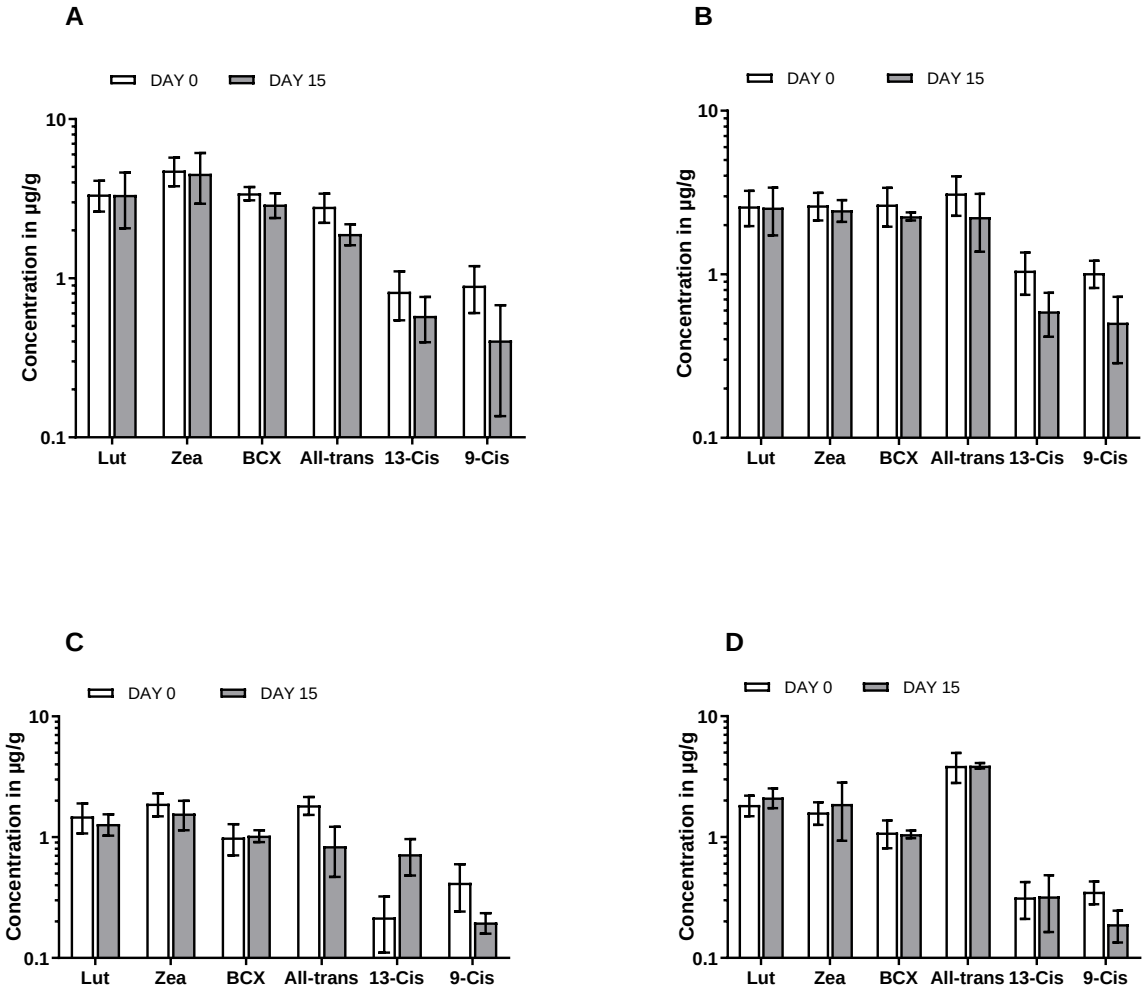



Figure 3-5. Stability of carotenoids in four different genotypes of maize (A) GV662, (B) GV 664, (C) GV665 and (D) GV 672 after 15-day storage at -20°C. Error bars represent standard deviations (n = 3).

## 3.5 Discussion

It is well established that biofortification of staple crops enriched with provitamin A carotenoids can be used as a tool for sustainable and cost-effective food-based solutions in combating micronutrient deficiencies (Dube et al., 2018). However, an important aspect to consider when assessing the effectiveness of any biofortification strategy is the potential effect of food processing on the final provitamin A content in the food products as this is what will

potentially present amounts accessible for vitamin A activity to the consumer. Moisture content based on dry matter was calculated on the processed food samples to make it easier in estimating carotenoid and vitamin E content on a dry weight basis. Results of the dry matter content reported in this study are similar to those reported in previous studies (Bengtsson et al., 2008; Pillay et al., 2014; Ooko Abong et al., 2020). An average of 30% dry matter is within the desired breeding targets of biofortified food crops while the dry matter of anything above 35% has been associated with severe reductions in both yield and quality of the bred crops (Bengtsson et al., 2008).

### 3.5.1 Baseline carotenoid content

The distribution and quantification pattern of total carotenoids in the biofortified food samples shows that zeaxanthin was the dominant carotenoid in all the 4 genotypes of maize. This was followed by all-*trans*-β-carotene accounting for between 50 to 95% of total carotenoids depending on the genotype analysed. Results of our studies are consistent with previous studies (Mugode et al., 2014; Pillay et al., 2014; Muzhingi et al., 2017) that evaluated the retention of provitamin A carotenoids in biofortified maize hybrids prepared under local conditions and found the non-provitamin A carotenoids in higher concentrations in comparison to the provitamin A carotenoids. The xanthophyll zeaxanthin has further been reported to provide protection from macular degeneration (Calvo-Brenes and O'Hare, 2020) and together with all-*trans*-β-carotene provide orange colour of maize (Pillay et al., 2014). All-*trans*-β-carotene is known to be a precursor of vitamin A with the highest activity (Green and Fascetti, 2016; Demiray and Tulek, 2017).

Similar findings on provitamin A carotenoids content in studies involving biofortified food crops have been reported (Muzhingi et al., 2008; Mugode et al., 2014; Pillay et al., 2014; Dube et al., 2018; Taleon et al., 2019). As expected, no carotenoids were detected in the local white variety of maize which also served as a control in this study. The baseline content for β-cryptoxanthin of 2.98 µg/g DW was slightly higher than previously reported figures of 1.73 ± 0.79 (Li et al., 2007) and 1.60 ± 0.21 µg/g DW (Muzhingi et al., 2008). In contrast, a study by Pillay et al. (2014) and Mugode et al. (2014) reported a range of β-cryptoxanthin values that were considerably higher than our results at 3.7 – 4.8 µg/g DW and 4.4 – 6.26 µg/g DW respectively. The observed differences could be explained by genetic variations in the maize varieties whose effect and diversity has reportedly proved useful in the conventional breeding of provitamin A biofortified maize (Muzhingi et al., 2017). Expectedly, the concentration of the β-carotene isomers were low. This is usually so in raw food samples as the minimal stress

applied during sample preparation in unprocessed foods may not cause significant *trans-cis* isomerisation.

### 3.5.2 Effect of processing on carotenoid content

In the current study, cooking maize mealie meal into *Nshima* but not *Nshima meal* resulted in reduced content of carotenoid in biofortified maize varieties. This finding is in agreement with previous observations (Rodriguez-Amaya and Kimura, 2004; Li et al., 2007; Mugode et al., 2014; Rodriguez-Amaya, 2015) where the effect of high temperature was demonstrated to negatively affect carotenoid content. In the said study, high temperatures with prolonged time resulted in the degradation of carotenoids. These results are however in contradiction to other studies (Muzhingi et al., 2008; Pillay et al., 2014; Diaz-Gomez et al., 2017; Taleon et al., 2017; Dube et al., 2018) who in their separate studies found that processing led to apparent increased carotenoid content. These studies attributed the increase in content to increased extractability of carotenoids arising from the disruption of protein structures holding the carotenoid in their natural environment. Interestingly, Mugode et al. (2014) found that maize processing reduced the content of all-*trans*-β-carotene but not the xanthophylls, β-cryptoxanthin, lutein and zeaxanthin. While losses in content can be attributed to heat exposure and high moisture content which ultimately reduces dry matter, the variations in results from one study to the other could be as a result of different food processing methods, carotenoid extraction methods and genotype differences in the maize samples (Ortiz et al., 2016). The processing method used in the current study was one where biofortified maize meal was used to prepare *Nshima* and the composite meal in the form of *Nshima meal* while previous studies (Muzhingi et al., 2008; Diaz-Gomez et al., 2017; Mugode et al., 2014) assessed carotenoid in maize meal prepared as either porridge or *Nshima* on its own. Furthermore, Diaz-Gomez et al. (2017) assessed content on the basis of total carotenoid content which does not take into account differences in content of individual carotenoids and other possible *trans-cis* isomerisation of compounds. In most countries, foods rich in dietary carotenoids are consumed in a plethora of combinations (Rodrigues et al., 2017). In the current study, this composite meal consisting of *Nshima* and stew with vegetable cooking oil recorded significant increases in the content of all the carotenoids. The plausible explanation is that the meal preparation at high temperature caused the disruption of the cellular matrix in the food and thus facilitating carotenoid liberation into the partitioning solution. This is consistent with previous findings (Mayer-Miebach and Spiess, 2003; Kim et al., 2015b; Zhang et al., 2020) where an increased content in carotenoids after meal preparation was attributed to improved solubility in the lipid fraction.

In sub-Saharan Africa, traditional methods of sweet potato preparation include boiling, steaming, roasting and drying (Chilungo et al., 2019). In the current study, boiling and baking of OFSP were selected as the two processing methods on account of feasibility. Roasting as a processing method could not be done on OFSP as this cooking preparation method involves the use of an open area, a procedure that could not be undertaken within our laboratory facilities for safety reasons. Results showed that the *all-trans*- configuration of β-carotene dominated in all the OFSP genotypes with a mean total found to vary between 60.1 to 80.6 µg/g DW. These values correspond well with those previously reported (Van Jaarsveld et al., 2006; Kidmose et al., 2007; Júnior et al., 2020). Specifically, one study found a mean variability to be between 1,240 – 10,800 µg/100g (12.4 – 108 µg/g) (Van Jaarsveld et al., 2006). In another study, among the 100% of the total carotenoids present in the raw materials, 79.6% corresponded to all-t*rans*-β-carotene when quantifying carotenoid content of fresh sweet potatoes (Júnior et al., 2020).

Boiling and baking of biofortified OFSP led to reduced concentration of all-*trans*-β-carotene but increases in β-cryptoxanthin and the *cis*-isomers of β-carotene in all the varieties. This observation is consistent with literature findings on the effect of processing on provitamin A rich foods (Kidmose et al., 2007; Kim et al., 2015b; Nzamwita et al., 2017) where exposure to high temperature with prolonged time was demonstrated to result in the degradation of carotenoids on one part and lead to the formation of carotenoid isomers on the other. Structural alterations and non-enzymatic oxidation of carotenoids have been cited as possible factors that could further lead to reduced changes in provitamin A activity in sweet potato after exposure to different processing treatments (Rodriguez-Amaya, 2002; Rodriguez-Amaya, 2015). It is however not clear as to why processing caused increases in β-cryptoxanthin in the biofortified OFSP but this observation could be attributed to the reported thermal stability in hydroxylated carotenoids that includes this particular carotenoid. Previous studies (Saini and Keum, 2018) reported similar observations with hydroxylated carotenoids such as lutein and zeaxanthin that demonstrated greater thermal stability compared to carotenes upon exposure to thermal heat. In this study, 9-*cis*-β-carotene and 13-*cis*-β-carotene were the two main β-carotene isomers formed after processing of biofortified OFSP varieties. Though both β-carotene isomers were only found in trace amounts in the unprocessed samples, the corresponding amount formed was 2 and 4 times greater than the amount in unprocessed samples after boiling and baking methods respectively.

Interestingly, in our study like many other studies, the formation of 9-*cis*-β-carotene was lower than 13-*cis*-β-carotene. This was more pronounced in baked samples that on average had higher concentrations than similar quantities used during boiling. Previous studies have reported similar findings (Bengtsson et al., 2008; Nzamwita et al., 2017) who reported that the most intense isomerisation happened as a result of baking. In the current study, baking at 240°C for 20 to 30 minutes provided more intense heat in comparison to boiling, resulting in higher concentrations of the 13-*cis*-β-carotene. The intensity is probably due to high water losses as a consequence of simultaneous heat and mass transfer within the OFSP product and the environment inside the oven during baking process. In non-biofortified OFSP and BNSQ samples, like the observations made with biofortified samples, both boiling and baking led to significant losses in all-*trans*-β-carotene. In comparison to OFSP, BNSQ recorded greater losses of β-cryptoxanthin when baking was used as a processing method in the two food samples. The explanation for these losses could be either as a result of genotypic variations, differences in carotenoid substructure localization in the plant chromoplasts or the possibility of sample storage as these products where merely procured from the open market places. Previous studies have reported up to 16% loss in carotenoid content for foods that were left in the open air and thus exposed to both sunlight and oxygen (Bengtsson et al., 2008) resulting in the photo-degradation of compounds in these foods. On the aspect of localization, sweet potato carotenoids in chromoplast substructures are found bound in crystalline form with a few plastoglobules while carotenoids in BNSQ are found mainly in plastoglobules, tubules and vesicles of the chromoplast substructures (Dhuique-Mayer et al., 2018). It is these tubules and vesicles, containing protein-bound carotenoids in BNSQ that tend to act as barriers in limiting the release of carotenoids in BNSQ during processing that could possibly explain the observation in our experiment (Jeffery et al., 2012). It is also possible that the carotenoid composition and concentrations were different owing to the natural abundance of the genotypes used in the study.

When the effects of processing on the content of vitamin E in food samples was assessed, it was observed that the reduction in vitamin E content was more pronounced in complex meals prepared from biofortified maize varieties than was case with OFSP. It is not entirely clear why the degradation in vitamin E only affected maize varieties. The plausible explanation may relate to food matrix and preparation methods that may ultimately have affected the vitamin E antioxidant properties. Knecht et al. (2015) attributed the loss of vitamin E activity to two possibilities; first being the presence of α-tocopherol oxidase, found in a variety of plants, to

be the main driver of tocopherol degradation and secondly, low pH leading to reduced tocopherol oxidase activity. This may especially be true in our study as the meal prepared from *Nshima* had different components probably leading to low pH. Maize grain itself used in the preparation of *Nshima* has been described elsewhere as an acidic food (Majzoobi et al., 2016) that could have contributed to the lowering of the pH in the composite meal in the current study. Future studies will have to determine the direct effect of acidic and alkaline conditions on vitamin E.

Boiling and not baking led to increased individual carotenoid content except for the isomerised forms in all the OFSP genotypes analysed. One of the most important aspects of understanding the potential nutritional impact of a food article that undergoes any form of processing is the amount of the nutrient retained in the final product. Thus in retention studies, raw and processed samples are compared and analysed as paired samples in order to avoid sampling errors (Bengtsson et al., 2008). It is worth mentioning that for the purposes of systematic result comparisons, BNSQ and ordinary OFSP samples were not included in our retention studies. In the present study, retention of Pro-VITA carotenoids in the raw and processed biofortified maize and OFSP was calculated based on their dry weight and corrected for changes in moisture content due to the anticipated loss of soluble solids, loss or uptake of water and uptake of oil as the case may be. For biofortified foods, assessment of retention is important for these foods to retain sufficient provitamin A carotenoids for the benefit of the people who will consume them (Bechoff et al., 2018). In the present study, the retention of total Pro-VITA carotenoids was high in all the maize genotypes in the range of 67.7% to 158.5%. This observation is consistent with previous findings (Muzhingi et al., 2008) who observed carotenoid increase in the cooked yellow maize products. Despite processing causing losses in all-*trans*-β-carotene, the carotenoid with major vitamin A activity, subsequent formation of β-carotene isomers compensated for the loss as the retention of total Pro–VITA carotenoids was estimated. Previous studies (Mugode et al., 2014; Pillay et al., 2014; Sowa et al., 2017) have attributed the higher retention of carotenoids to better extractability from the food matrix. In contrast, the higher retention could as well imply deactivation of oxidative enzymes that would degrade carotenoids rather than true increases in the carotenoid content (Sowa et al., 2017). In the present study, an increase in carotenoid retention of 115.4% to 178.9% was further observed in the *Nshima* that was prepared from GV 665 and GV 672 maize genotypes. These results are comparable to retention values in biofortified maize varieties reported by Mugode et al. (2014) who, in his study showed retention values in the range of 110 – 127% in some biofortified

maize genotypes. For the present study, the lower total Pro-VITA retention observed in GV662 and GV665 could be explained by the high lutein and zeaxanthin contents in the 2 products that did not eventually contribute to the total Pro-VITA. In the composite meal, *Nshima meal*, retention of Pro-VITA was high in 3 of the 4 maize genotypes. This is not surprising as the composite meal had the lipid component in the preparation that likely could have increased solubility and therefore enhanced extractability during carotenoid analysis resulting in the apparent increase in their content.

In the biofortified OFSP varieties, the retention values in the present study ranged from 83.6 – 116.3% across OFSP genotypes and varied with processing conditions. These results are comparable to previous studies (Bengtsson et al., 2008; Van Jaarsveld et al., 2006) which found retention values of OFSP in the range of 77 - 92%. The minor differences could be attributed to genotype differences, processing conditions and methods of carotenoid extractions. Boiling had higher retention in comparison to baking. The fact that boiling results in high retention of Pro-VITA is in itself encouraging considering that this is the common method of processing for human consumption in the targeted regions for biofortified foods and expectedly, continued consumption would improve people's vitamin A status.

### 3.5.3 Stability of carotenoids at different storage temperatures

Considering that carotenoids are highly unsaturated molecules composed of many double bonds and therefore susceptible to oxidation and degradation from high temperature, presence of oxygen and light (Che et al., 2016), the present study assessed the stability of carotenoids when stored at -20°C for 15 days. The rationale was based on the premise that long-term storage is a normal part of domestic consumer's behaviour and in most cases, its good laboratory practice to freeze food samples prior to analysis in both cases for convenience and maintenance of sample integrity at -20°C (Dias et al., 2014). In the present study, freezing led to decreased content in all-*trans*-β-carotene and the associated isomers by an average of 50% after a 15 day storage at -20°C in the processed maize meal products. The xanthophyll carotenoids, lutein, zeaxanthin and β-cryptoxanthin decreased their content by an average of 10%, implying therefore that they were relatively more stable than the carotenes. This is in contrast to previous studies (Scott and Eldridge, 2005) whose results indicated that storage of corn at -20°C led to significant increases in zeaxanthin and lutein, representing the class of xanthophylls and decrease content in α-carotene that represents the group of carotenes. Another study (Dias et al., 2014) also found that storage of carotenoid rich foods for up to 11 weeks led to a significant

decrease in the concentration of α-carotene and zeaxanthin but not the other xanthophylls. This phenomenon has been attributed to the preservation of the enzymatic pathways for the biosynthesis of carotenoid flux in the xanthophylls (Sun et al., 2020). These results are suggestive that -20°C storage temperature is not ideal for long-term storage and preservation of carotenoids in food samples.

## 3.6 Conclusion

The content of provitamin A carotenoids in food samples is hugely affected by the type of processing used, type of carotenoid under investigation and initial content in the unprocessed samples. While biofortified food crops are conventionally bred to contain specific concentrations of carotenoids, it is important for institutions involved in breeding crops to deliberately set target concentrations for specific populations high enough to compensate for losses encountered during the processing procedures. In this case, genotypes with enhanced concentrations of non-provitamin A carotenoids in lutein and zeaxanthin may not serve their intended purpose if such food crops are meant to mitigate vitamin A deficiencies in afflicted populations.

Food processing and preparation techniques are important factors that ultimately reflect changes in the retention of the final product to be consumed, absorbed or extracted for analysis. In the present study, boiling instead of baking and preparation of the composite *Nshima meal* remarkably improved retention of the provitamin A carotenoids. Understanding the best food processing and preparation can therefore be used to help consumers derive maximum benefits from such practices. Theoretically, storage of food samples at temperatures below -80°C may be ideal for preservation and stability of carotenoids in foods but these storage facilities are not readily available or may come at a huge economic cost in both domestic and laboratory facilities. Future studies may need to be conducted to establish ideal storage temperatures and time that raw and differently processed food samples could be kept. While carotenoid content provides a good indication of what is contained in the food matrix, it is important to understand another potentially challenging aspect that concerns accessibility of these compounds during gastro-intestinal digestion. Therefore, the next chapter describes different digestion systems and how these models are able to closely simulate the human digestion system to aid our understanding of carotenoid uptake and bioavailability.

# CHAPTER 4

# Fate of dietary carotenoids in different simulated gastrointestinal digestion models using test foods of varying caloric densities

## 4.1 Summary

The impact of food composition on carotenoid stability and their delivery for intestinal absorption was investigated. Using a combination of *in vitro* digestion protocols as proposed by the INFOGEST network, samples of goji berries, kale and test foods prepared from biofortified maize (*Nshima*) and orange-fleshed sweet potato (OFSP) were sampled at different stages of digestion and analysed using RP-HPLC. Results of the average recoveries of carotenoids were around 90% at the oral phase, around 40% after the oral + gastric phase and around 55% after oral + gastric + intestinal phase for all-*trans*-β-carotene. Owing to the low concentrations of carotenoids in other test foods, only OFSP containing high provitamin A carotenoids with the following formulations: (i) OFSP (ii) OFSP + LIPID (5% w/w) (iii) OFSP + LIPID (5% w/w) + PROTEIN (5% w/w) and equalised by meal volume were digested. The addition of 5% (w/w) LIPID led to OFSP led to a two-fold increase in the bioaccessibility of all-*trans*-β-carotene (25 to 49%) and 30 to 57% for β-cryptoxanthin. Further addition of 5% PROTEIN (w/w) to the (OFSP + LIPID (5%) formulation led to an increase in the caloric density and thus delayed delivery to the intestinal phase and reduced bioaccessibility for both carotenoids. Results of this study suggest that dietary modification, through food composition, can be used to deliver sufficient carotenoids for uptake and possible absorption to improve vitamin A body stores in humans.

*Keywords*: Bioaccessibility, carotenoids, semi-dynamic, OFSP, calories, digestion

## 4.2 Introduction

Food based strategies, particularly consumption of foods rich in provitamin A carotenoids can ameliorate the challenges of vitamin A deficiency. However, to exert their bioactivity, carotenoids need to be released from the food matrix and be absorbed by the body after digestion. The process of carotenoid absorption is complex involving several stages including (i) release from the food matrix, (ii) incorporation into mixed micelles, (iii) uptake by intestinal epithelial cells, (iv) metabolising into vitamin A or packaging into chylomicrons and secretion into the lymphatic system (Goltz et al., 2012) to the site of action or storage. The first two are the critical stages that not only define bioaccessibility but also provide fractional estimates of the absorbable compounds. In essence, their release from the food matrix, solubilisation in the lipid droplet and subsequent dispersion within the gastrointestinal tract during simulated digestion, has been shown to be a reliable predictor of their bioavailability in humans (Reboul et al., 2006).

While human studies represent the gold standard for understanding food digestion, *in vitro* methods mimicking the human digestion process have been applied to determine bioaccessibility of nutrients and to assess the digestion of foods (Petry and Mercadante, 2017). These methods have the advantage over *in vivo* methods as they do not require ethical approval, are less variable and sampled at different stages during digestion and are less costly (Rodrigues et al., 2016; Petry and Mercadante, 2017; Rodrigues et al., 2017). However, different *in vitro* digestion methods have been used, each with varying results and thus making it difficult to ascertain which model provides the most accurate reflection of carotenoid bioaccessibility values that may closely relate the *in vivo* situation. Furthermore, carotenoids have been reported to have very low bioaccessibility and bioavailability relative to other fat and fat-soluble compounds like omega-3-fatty acids and tocopherols (Borel et al., 2013). Some authors have in fact suggested that up to 25% of β-carotene is lost during the gastric stage of digestion while 40% and 30% of lutein and lycopene, respectively, are lost during the jejunal phase (Kopec et al., 2017). Their sensitivity to various GI tract conditions such as pH and pro-oxidative species have been cited as the major reason for their instability (Halliwell et al., 2000; Kanner and Lapidot, 2001). Secondly, food is consumed in a plethora of combinations and as such, comes in different structures and compositions. Recent data suggests that structure and composition of food has an impact on nutrient absorption and digestion (Mulet-Cabero et al., 2019). Food structural features, specifically composition, meal size and caloric content alter gastric

behaviour and therefore impact on nutrient delivery and digestion in the small intestines (Mulet-Cabero et al., 2017; Thuenemann et al., 2015). However, the kinetics of gastric digestion and its effects on delivery and bioaccessibility of dietary carotenoids from test foods are poorly understood. To the best of our knowledge, no study has investigated the kinetics of carotenoid delivery from the simulated gastric phase into the small intestines from a biofortified OFSP test meal, prepared using traditional processing methods, with varying amounts of calories. A further challenge noted from previous studies is the interpretation of bioaccessibility data, differences in gastrointestinal conditions and digestion models used (Bourlieu et al., 2015). Until physiologically relevant *in vitro* conditions across different digestion models are consistently applied to mimic *in vivo* conditions, comparisons with human studies will continue to produce varying results. Given this background, the present study had two major objectives:

I. To compare bioaccessibility of carotenoids from different food matrices of varying carotenoid composition using the *in vitro* static and semi-dynamic digestion models as described by the INFOGEST network.
II. To investigate the impact of food composition on the bioaccessibility of carotenoids in composite meals prepared from biofortified foods as affected by caloric content and gastric digestion kinetics.

### 4.2.1 Experimental procedures

Goji berries, kale, biofortified OFSP and *Nshima* prepared from biofortified maize meal were used in both static and semi-dynamic digestion models to assess the bioaccessibility of carotenoids. These test samples were carefully selected to provide different structure, energy and composition differences which would impact on both digestion and nutrient absorption. Composite meals, prepared from OFSP and *Nshima* meal, as described in **section 2.2.2**, were used only in the semi-dynamic model to investigate the role meal composition plays in modulating gastric digestion, emptying and ultimately, bioaccessibility of carotenoids. Samples were collected from different (oral, gastric and intestinal) phases of digestion, analysed and quantified using HPLC-DAD methods described in **section 2.4.11**. The *in vitro* digestions were then carried out according to the protocol proposed by COST Action INFOGEST network and the recently developed semi-dynamic models (Minekus et al., 2014; Mulet-Cabero et al., 2020a) with adaptations for carotenoid analysis (Rodrigues et al., 2016) as shown in Figure 4-1 of the schematic diagram.

### 4.2.2 Statistical analysis

The statistical analyses were conducted using GraphPad prism 8.0 software on three replicate samples. The means of carotenoid bioaccessibility within the same method of digestion were compared by one-way ANOVA followed by Tukey's post-hoc test. The means of carotenoid percent retention and bioaccessibility for the same carotenoid in a given sample determined by the different digestion methods were compared by Student's *t* test for two independent means. Differences were stated significant at $P < 0.05$.

## 4.3 Results

### 4.3.1 Bioaccessibility of dietary carotenoids

The major carotenoids found after *in vitro* digestion of *Nshima*, OFSP, kale and goji berries were all-*trans*-β-carotene, β-cryptoxanthin, lutein, zeaxanthin, 13-*cis*-β-carotene and 9-*cis*-β-carotene (Table 4-1). Following intestinal digestion, the bioaccessibility of provitamin A carotenoids all-*trans*-β-carotene, β-cryptoxanthin and the β-carotene isomers were highest in OFSP and lowest in *Nshima* while greater amounts for lutein xanthophyll were recorded in Kale and *Nshima*. The relative bioaccessibility of provitamin A carotenoids ranged from 7.9 – 115.1% using the static method and 11.4 – 121.8% with the semi-dynamic model. In the case of xanthophylls, the relative bioaccessibility ranged from 41.7 – 84.9% and 43.9 – 98.6% using the static and semi-dynamic methods respectively. By comparison, the efficiency of micellarisation of all-*trans*-β-carotene, a carotenoid with the greatest provitamin A activity, was significantly greater using the semi-dynamic (66.0 ± 2.0%) compared to the static method (58.0 ± 5.0%) ($P < 0.05$). Similarly, the micellarisation efficiency was greatest for lutein when assessed with the semi-dynamic method (75.3 ± 6.2%) compared to the static method (60.2 ± 7.1%) ($P < 0.05$). No significant differences were detected for 9-*cis* and 13-*cis*-β-carotene isomers in the two digestion models (Table 4-1).

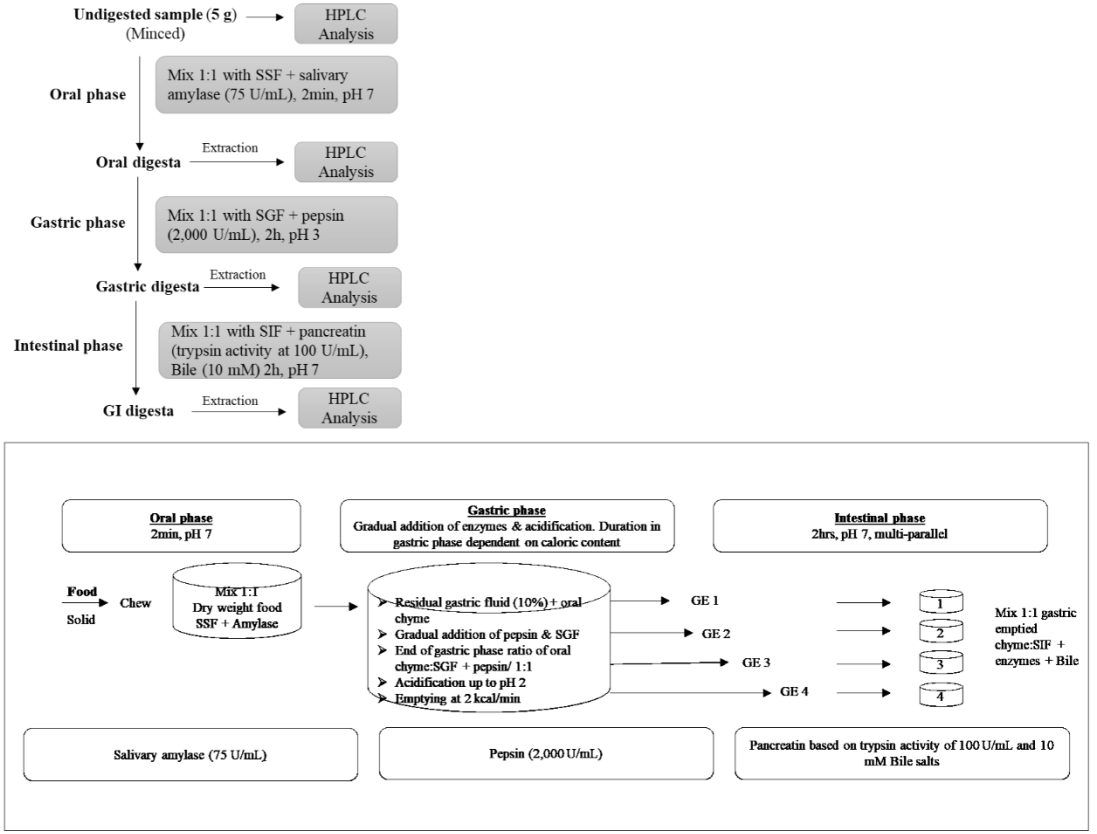



Figure 4-1: Overview and schematic diagram of the static (**top section**) and semi-dynamic (**bottom section**) *in vitro* digestion models. Enzyme activities calculated in Units per mL and GE are gastric emptying points.

Table 4-1: Bioaccessibility of carotenoids in test foods simulated following *in vitro* static and semi-dynamic gastrointestinal digestion.

| | | | Bioaccessibility (%) | |
|---|---|---|---|---|
| **Carotenoid** | **Food sample** | **Starting Conc. (µg/g)** | **Static** | **Semi-dynamic** |
| Lutein | *Nshima* | 1.58±0.06 | ***55.1 – 65.2$^{b}$*** | ***70.9 – 79.7$^{a}$*** |
| | OFSP | ND | - | - |
| | Kale | 11.27±3.01 | ***64.6 – 81.5$^{b}$*** | ***83.5 – 84.6$^{a}$*** |
| | Goji berries | ND | - | - |
| Zeaxanthin | *Nshima* | 1.39±0.16 | 41.7 - 84.9$^{a}$ | 43.9 – 98.6$^{a}$ |
| | OFSP | 0.15±0.04 | - | - |
| | Kale | 3.73±0.96 | 52.0 – 55.8$^{a}$ | 57.9 – 66.0$^{a}$ |
| | Goji berries | ND | - | - |
| β-cryptoxanthin | *Nshima* | 1.06±0.08 | 20.8 – 35.8$^{a}$ | 27.4 – 33.0$^{a}$ |
| | OFSP | 4.28±0.54 | 55.1 – 72.9$^{a}$ | 66.8 – 78.9$^{a}$ |
| | Kale | ND | - | - |
| | Goji berries | 22.33±3.37 | 38.0 – 46.0$^{a}$ | 32.8 – 57.1$^{a}$ |
| All-*trans*-β-carotene | *Nshima* | 3.69±0.49 | 7.9 – 12.2$^{a}$ | 11.4 – 16.8$^{a}$ |
| | OFSP | 80.6±3.10 | ***54.2 – 61.8$^{b}$*** | ***64.6 – 67.4$^{a}$*** |
| | Kale | 6.03±0.71 | 33.3 – 46.9$^{a}$ | 35.7 – 52.2$^{a}$ |
| | Goji berries | 22.53±1.05 | 30.5 – 39.5$^{a}$ | 29.9 – 48.2$^{a}$ |
| 13-*cis*-β-carotene | *Nshima* | 0.83±0.17 | - | - |
| | OFSP | 4.36±0.88 | 80.7 – 115.1$^{a}$ | 82.8 – 115.4$^{a}$ |
| | Kale | 1.33±0.40 | 86.5 – 101.5$^{a}$ | 76.7 – 121.8$^{a}$ |
| | Goji berries | 1.53±0.40 | 88.2 – 105.2$^{a}$ | 85.6 – 101.3$^{a}$ |
| 9-*cis*-β-carotene | *Nshima* | 0.92±0.35 | ND | - |
| | OFSP | 1.16±0.38 | 87.1 – 107.8$^{a}$ | 96.6 – 104.4$^{a}$ |
| | Kale | 0.7±0.2 | ND | - |
| | Goji berries | 0.53±0.32 | ND | - |

Data are mean ± SD (n=3). Bioaccessibility indicates the percentage of the carotenoid in the food that was detectable in the digesta at the end of the small intestinal phase in both models. Different lowercase letters in the same row indicate significant differences ($P < 0.05$). ND = not detectable.

### 4.3.2 Meal composition and its effect on digestion

The gastric residence time was calculated on the basis of meal size, composition and caloric content and thus, the present study used nutrient composition whose estimates are described under **section 2.2.3**. Using these parameters on the basis of nutritional composition described in Table 4-2, the energy content for OFSP, *Nshima*, Kale and Goji berries was calculated as 0.263, 1.154, 0.583 and 0.666 kcal/mL. Given this background, the gastric residence time for OFSP, *Nshima*, kale and goji berries was found to be 44, 192, 96 and 112 mins respectively (Figure 4-3). With these gastric residence times, the total emptying rates were 1.09 mL/min for OFSP, 0.25 mL/min for *Nshima*, 0.49 mL/min for Kale and 0.43 mL/min for goji berries. It is

worth mentioning here that the food composition parameters served as a guide in estimating duration of gastric digestion and the calculation of the gastric emptying points. For this experiment, and because of the high levels of provitamin A carotenoids in OFSP, only composite meals prepared from this test food were used to evaluate the effects of digestion on modulation of carotenoid absorption.

Table 4-2: Nutritional composition of food samples subjected to both static and semi-dynamic *in vitro* digestion models on dry weight basis.

| | **OFSP** | **Nshima** | **Kale** | **Goji berries** |
|---|---|---|---|---|
| Carbohydrate, g | 20.7 | 84 | 8.75 | 28 |
| Fat, g | 0.15 | 4 | 0.63 | 1 |
| Protein, g | 2.01 | 8 | 4.4 | 28 |
| Energy, kcal | 92.2 | 404 | 58.3 | 233 |
| Weight of meal, g | 350 | 350 | 350 | 350 |
| Kcal/mL | **0.263** | **1.154** | **0.583** | **0.666** |

The energy content (kcal/mL) was different for each food item and was calculated using the Atwater factors of 9 kcal/g for fat and 4 kcal/g for protein and carbohydrates respectively.

### 4.3.3 Carotenoid stability in different phases of *in vitro* static digestion

The aim in this section was to assess the stability of carotenoids in different phases of the *in vitro* static digestion model. Consideration was given to two carotenoids; all-*trans*-β-carotene and β-cryptoxanthin provitamin A carotenoids partly because of their nutritive effect and also that some of the samples, OFSP and *Nshima* from maize in particular, were biofortified material with enhanced provitamin A that was later used in the preparation of complex test foods in understanding the effects of GI digestion in modulating the bioaccessibility of provitamin A carotenoids as described under **section 4.2.5.** The carotenoid recoveries, herein referred to as stability after the oral, gastric and intestinal phases, were reported and represent the relationship between content at a particular phase of digestion and the content in food samples before digestion, given as a percentage. Carotenoid recovery was high in the oral digesta for both food samples under investigation resulting in mean percent recoveries of 90.9 ± 1.7% and 95.5 ± 3.6% for all-*trans*-β-carotene in *Nshima* and OFSP respectively. Similar results were observed in the same foods with mean recoveries of 92.3 ± 2.0% and 95.3 ± 1.3% for β-cryptoxanthin.

However, low percent recoveries for both carotenoids were observed in the two food samples during gastric digestion. In comparison to the hydrocarbon all-*trans*-β-carotene, β-cryptoxanthin exhibited greater stability with significantly higher mean percent recoveries of 52.3 ± 3.4% against 34.7 ± 2.0% in *Nshima* and 60.7 ± 1.7% against 40.0 ± 2.9% in OFSP (*P*

< 0.05). A slight but significant increase in the stability of both carotenoids was later observed after the intestinal phase. The mean percent recoveries for all-*trans*-β-carotene were 45.3 ± 2.1% in *Nshima* and 64.0 ± 4.0% in OFSP ($P < 0.05$). A similar trend with higher recoveries of β-cryptoxanthin were observed with mean percent recoveries of 70.0 ± 3.0% in *Nshima* and 76.3 ± 2.5% in OFSP.

In summary, average recoveries of carotenoids at the different phases of the *in vitro* static digestion was around 90% at the oral phase, around 40% after the oral + gastric phase and around 55% after oral + gastric + intestinal phase for all-*trans*-β-carotene. Similarly, the β-cryptoxanthin average recoveries were 94% at the oral phase, around 57% after the oral + gastric phase and around 73% after oral + gastric + intestinal phase. The increase at the intestinal phase could be attributed to the stabilisation of the micellar fraction brought about by the presence of biliary salts, lipid digestion products and pancreatic enzymes. In comparison to all-*trans*-β-carotene, β-cryptoxanthin, was the most stable carotenoid in both food samples and at all stages of digestion. Owing to either their low concentrations or absence in the selected biofortified samples of interest, β-carotene isomers, lutein and zeaxanthin were excluded in this and further experiments that followed.

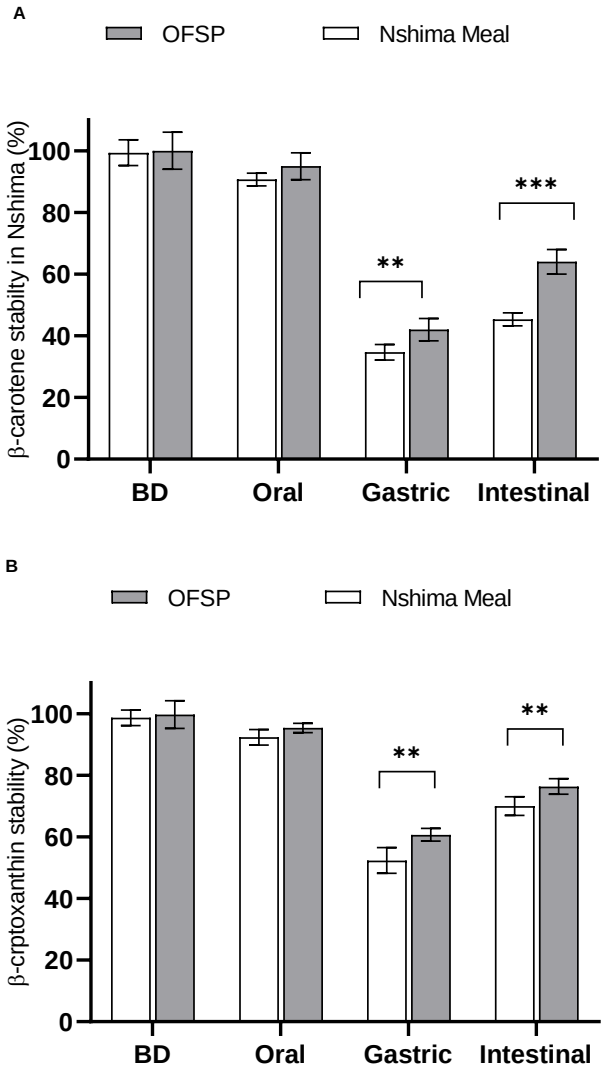



Figure 4-2: Stability of (A) β-carotene and (B) β-cryptoxanthin from Nshima and OFSP using the Static *in vitro* digestion method. Data are means ± SD, ($n$ = 3). Significance set at $P < 0.05$.

### 4.3.4 Relationship between caloric density, gastric emptying and carotenoid stability

In determining the extent to which dietary carotenoids are protected against destruction or loss during gastric digestion, the content, representing stability of all-*trans*-β-carotene and β-cryptoxanthin in OFSP with different caloric densities was characterised at each gastric emptying point after simulated adult gastric digestion conditions. In this regard, gastric residence time was estimated from caloric value of the test foods used while each GE point was used to collect digesta and establish carotenoid content to represent stability. Results were recorded as percentages on weight/weight basis as shown in Figure 4-2. For the purposes of standardisation, only OFSP with the following formulations were used; (i) OFSP (ii) OFSP + LIPID (5% w/w) (iii) OFSP + LIPID (5% w/w) + PROTEIN (5% w/w) and equalised by meal

volume (Table 4-3). The use of these food formulations, whose preparation details are discussed in **section 2.2.3** was purposefully planned to deliver similar amounts of carotenoids whose difference could only be determined by caloric composition. The effect of increasing caloric densities at constant weight was however meant to have a test meal that could progressively slow meal emptying rates, an effect most apparent with the larger meals (Moore et al., 1984). This is important in attributing the rate of gastric emptying to one parameter under investigations. To keep things simple, GE time points were deliberately set to four for each food digesta.

It is clear from the results of this experiment that the higher the caloric value, the longer the gastric residence time of each food sample. On its own, OFSP with a theoretically estimated caloric value of 0.263 kcal/g took 44 mins to complete gastric digestion. When 5% (w/w) lipid was added to the OFSP, its caloric value was raised to 0.388 kcal/g and hence the gastric residence time increased to 64 mins. A further addition of 5% (w/w) protein to the OFSP that already had 5% lipid increased both the caloric value from 0.388kcal/g to 0.494 kcal/g and residence time to 82 mins (Table 4-3). The stability pattern of recoveries after the first emptying point was similar in all the 3 samples investigated, with β-cryptoxanthin, demonstrating greater stability than all-*trans*-β-carotene. In the OFSP test food, the stability of all-*trans*-β-carotene was at an average of 85.0% after 11 mins compared to 71.5% and 68.0% for the OFSP+LIPID and OFSP+LIPID+PROTEIN after 16 and 20.5 mins, respectively. At the second GE point, the stability of all-*trans*-β-carotene was reduced to 64.0% in the OFSP test food after 22 mins with corresponding percentage reductions of 55.0 and 46.5% in OFSP+LIPID and OFSP+LIPID+PROTEIN after 32 and 41 mins. The effect of caloric content on stability and GE produced similar results for β-cryptoxanthin, though, on comparison basis, they were significantly higher than the results obtained with all-*trans*-β-carotene for each of the 4 GE aliquots in all the food samples ($P < 0.05$). At the first GE, in the case of β-cryptoxanthin, the digestive stability was 94.0% after 11 mins compared to 80.5 and 72% after 16 and 20.5 mins for OFSP+LIPID and OFSP+LIPID+PROTEIN, respectively. Similarly to the changes observed with all-*trans*-β-carotene at GE 3, β-cryptoxanthin showed approximately 36.0% reduction in OFSP after 33 mins, 51.0% reduction in OFSP+LIPID after 48 minutes and a 60.0% loss in OFSP+LIPID+PROTEIN after 61.5 minutes. Interestingly, there was a slight increase in the digestive stability of both carotenoids in the last GE aliquot in relation to the third GE in all the samples. Generally, the results of this experiment indicated that, OFSP with the lowest caloric density (0.263 kcal/mL) achieved complete gastric emptying after only 44

mins followed by both OFSP+LIPID (0.388 kcal/mL) after 64 mins and OFSP+LIPID+PROTEIN (0.494 kcal/mL) that achieved complete emptying after 82 mins. It should be noted that the use of GE 1, 2, 3 and 4 in the figures is for illustration purpose only as the actual residence times differ and are indicated in minutes.

Table 4-3: Nutritional composition of OFSP with different caloric values subjected to semi-dynamic *in vitro* digestion.

| | **OFSP** | **OFSP+LIPID** | **OFSP+LIPID+PROTEIN** |
|---|---|---|---|
| Carbohydrate, g | 20.7 | 20.7 | 20.7 |
| Fat, g | 0.15 | 5 | 5 |
| Protein, g | 2.01 | 2.01 | 5 |
| Energy, kcal | 92.2 | 134.49 | 150.49 |
| Weight of meal, g | 350 | 350 | 350 |
| Kcal/mL | **0.263** | **0.384** | **0.430** |
| Gastric Res. Time (min) | 44 | 64 | 72 |

### 4.3.5 Effect of caloric density on carotenoid bioaccessibility

The delivery of carotenoids to the small intestines was highest regardless of the caloric density during the first two GE points which also resulted in higher bioaccessibility values for both carotenoids. On the other hand, there was a general decrease in the carotenoid content from GE 1 up to GE 4 only to have a slight increase at the last emptying point. On the relationship between caloric density and bioaccessibility, the addition of 5% lipid to OFSP led to a two-fold increase in the bioaccessibility of both carotenoids (25 to 49%) for all-*trans*-β-carotene and 30 to 57% for β-cryptoxanthin. Further addition of 5% protein led not only to an increase in the caloric density but also to delayed delivery to the intestinal phase and reduction of the bioaccessibility in both carotenoids when compared to the OFSP+LIPID bioaccessibility values. As expected, OFSP+LIPID showed higher bioaccessibility values for both carotenoids at all the GE points.

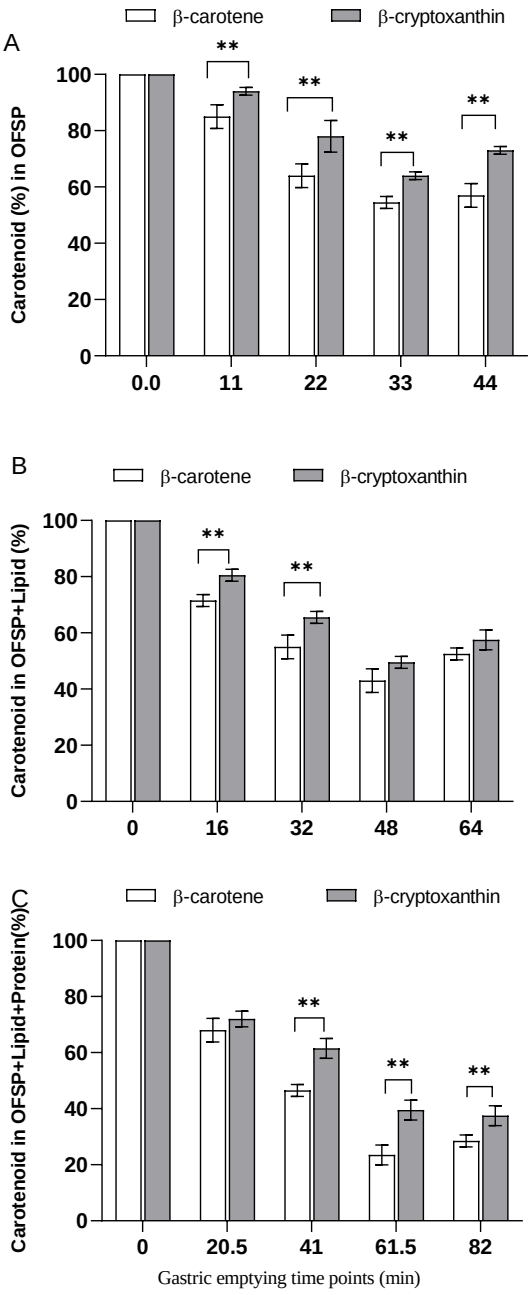



Figure 4-3: Carotenoid content in (A) OFSP, (B) OFSP+LIPID, (C) OFSP+LIPID+PROTEIN at each gastric emptying (GE) aliquot. Data are stability means percent values estimated on (w/w) ± SD (*n*=2). Significance set at $P < 0.05$.

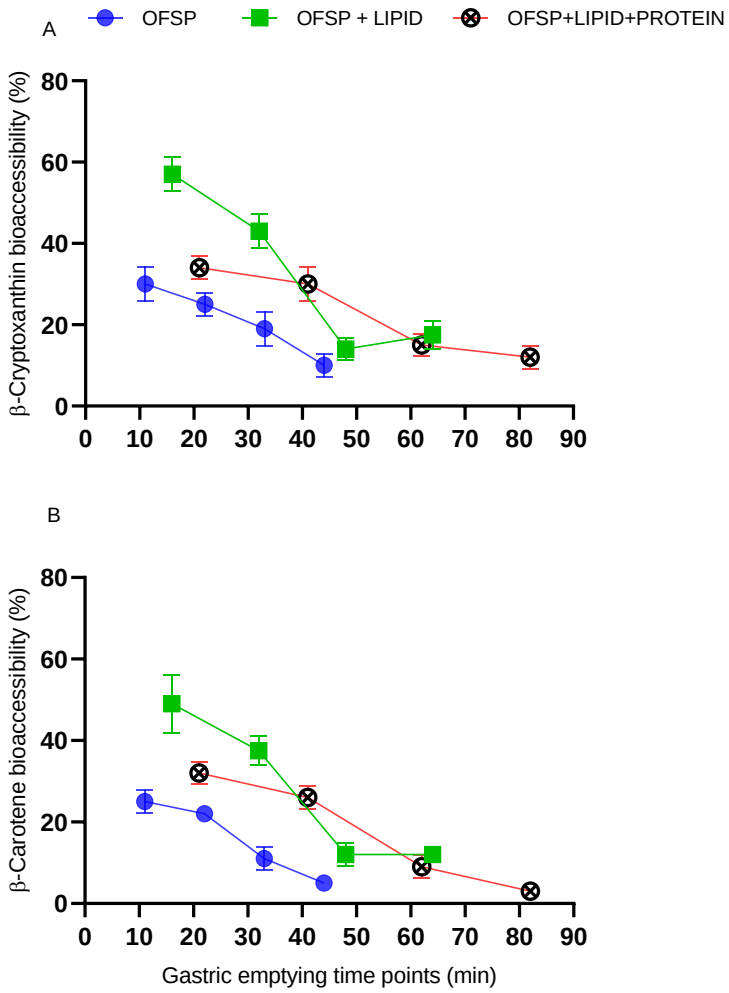



Figure 4-4: Bioaccessibility after GI digestion of (A) β-Carotene and (B) β-Cryptoxanthin in OFSP with different caloric values. Data are means ± SD ($n$ = 2).

## 4.4 Discussion

### 4.4.1 Stability of carotenoids during *in vitro* digestion

Direct determination of carotenoid content in food samples does not in itself provide sufficient information to predict their potential bioavailability *in vivo* (Santos et al., 2019). This is particularly true for compounds that are not stable and easily degradable under strong acidic or alkaline GI conditions. In order to assure bio-efficacy of bioactive compounds like dietary carotenoids upon food consumption, adequate and validated screening methods are required to provide estimates of what may potentially be available for absorption after digestion. In the present study, percentage recovery results obtained by use of the static harmonised INFOGEST model were lower than those obtained from the semi-dynamic model. This finding is not

surprising as the semi-dynamic digestion model employs physiologically relevant conditions such as gradual addition of fluids, acids and emptying after digestion and therefore likely to produce results in acceptable ranges compared to those obtained *in vivo*. Previous studies found that an adaptation of the semi-dynamic model, using simulations based on *in vivo* data, provided a close simulation of structural changes in the gastric phase of two dairy foods when compared with the human stomach (Mulet-Cabero et al., 2017). The static digestion model, like most *in vitro* digestion models, is simple to use, can easily be replicated and is devoid of inter-individual variations. It's major limitation apart from the obvious influence of the host-related factors such as gut health, nutritional status and genetic variations in humans, is the lack of complex body and food interaction that has been noted as a drawback of *in vitro* simulations (Minekus et al., 2014). Considering that the present study was dealing with complex meals, and the fact that *in vitro* static digestion models are routinely used to assess the bioaccessibility of carotenoids and other dietary lipophilic compounds (Rodrigues et al., 2017), there was a need to test the hypothesis that the semi-dynamic digestion model, on a comparative basis, produces bioaccessibility results that are significantly higher than those obtained through the static digestion model. It is only through such comparisons that valuable information on the rate and extent of the bioaccessibility of carotenoids, providing a more accurate measure of their bioavailability, would be obtained. For this reason, assessment of the performance of the digestion models was centred on the percentage recoveries after digestion. Furthermore, all carotenoids occurring in small quantities in the food samples including the non-provitamin A were excluded from the follow-up experiments. This is because of the challenge presented with detection limits after dilution of samples containing these compounds with simulated GI fluids. For this reason, lutein, zeaxanthin and the β-carotene isomers were not characterised beyond this stage. This therefore meant that only *Nshima* and OFSP were analysed using the static digestion method with test foods prepared from OFSP proceeding to be used in the semi-dynamic digestion method after favourable stability results.

In the analysis of carotenoids in OFSP and *Nshima* food samples during GI digestion, high carotenoid recoveries were recorded from the oral phase in both digestion models. Authors from previous studies have attributed this finding to the protective effect of their natural environment as there is very limited disruption at this stage of digestion and thus reducing interaction with the digestive fluids and enzymes whose activity may enhance their liberation from the food matrix (Petry and Mercadante, 2017).

A significant drop in the recoveries of carotenoids was observed at the gastric phase of digestion in both models. Recovery was particularly low for all-t*rans*-β-carotene which is in agreement with previous findings by Courraud et al. (2013) who, despite working with pure β-carotene, noted a 69% loss after the whole digestion process with the greatest loss mainly occurring in the gastric phase. Using the dynamic digestion model, Blanquet-Diot et al. (2009) reported a 30% reduction in β-carotene concentration after GI digestion while Courraud et al. (2013) reported a 50% reduction after the gastric phase. The drop in recoveries could be explained by the low pH levels in the gastric phase as carotenoids have been reported to be unstable in acidic and alkaline conditions (Rodriguez-Amaya, 1999; Rodriguez-Amaya, 2015). The recoveries later improved in the intestinal stage of digestion for both methods. The plausible explanation for this is that, carotenoids in the digesta from the gastric phase are stabilised at the intestinal stage through pancreatic and bile salts which aid in the formation of micelles resulting in higher accessible compounds. Furthermore, it is in the micellar fraction that the presence of other fat-soluble nutrients like vitamin E may provide the protective effect to compounds of similar structure (Alminger et al., 2014). In contrast, studies by Chitchumroonchokchai et al. (2004) and Blanquet-Diot et al. (2009) did not observe any change in the stability of lutein, a xanthophyll carotenoid over the course of digestion. It is not entirely clear as to why there is this divergence in results but may be due to the different food matrices and food preparation methods used.

In the present study, β-cryptoxanthin was more stable than the hydrocarbon all-*trans*-β-carotene throughout the digestion process. This finding is in agreement with previous studies (Blanquet-Diot et al., 2009; Courraud et al., 2013; Petry and Mercadante, 2017; Rodrigues et al., 2017; Rodrigues et al., 2016) who all seem to attribute the stability of the carotenoids to the structural features of the compound. In their study, Tyssandier et al. (2001) support this claim by suggesting that luminal stability of the carotenoids are largely dependent on their hydrophobicity. They demonstrated in their study that the percentage of carotenoids transferred from the emulsion lipid droplet to the mixed micelle was inversely proportional to carotenoid hydrophobicity with lutein > β-carotene > lycopene (Kopec et al., 2017).

The present study did not observe major changes in the concentration of 9-*cis* and 13-*cis*-β-carotene after digestion, which in itself implies that isomerisation did not take place in significant amounts. This finding is in agreement with previous studies (Ferruzzi et al., 2006; Kopec et al., 2017) who, upon using *in vitro* static digestion demonstrated that the percentage *cis*-isomer contribution to total carotenoid content is well maintained for β-carotene. Other

studies (Granadolorencio et al., 2007; Courraud et al., 2013) could not detect the β-carotene isomers after digestion supporting the hypothesis that very little isomerisation takes place during GI digestion.

### 4.4.2 Effect of food composition on gastric emptying and carotenoid bioaccessibility

One of the fundamental principles of digestion is that the process itself is dynamic and not at all static. The implication of this statement is that during the process of digestion, food entering the GI tract will be transferred from one compartment to another at variable rates depending on its structure, caloric content, osmolality and rheological properties (Dupont and Mackie, 2015). On the basis of this fact, it can be agreed with previous studies suggesting that food structure and its composition can facilitate or delay the release of nutrients during digestion and their absorption by the human body (Mat et al., 2016). With the understanding that gastric emptying is a complex process that depends on a number of factors, chiefly being the caloric density of a meal, that regulates the rate of gastric emptying (Calbet and MacLean, 1997), the present study used similar test meals that differed in caloric density but equalised by volume to study the kinetics of both, carotenoid delivery and bioaccessibility. The model of the present experiment that the calorie dense test foods emptied slower than test foods with lower caloric density, had less stable compounds and reduced carotenoid bioaccessibility after the entire course of GI digestion was based on previous studies by Velchik et al. (1989), who in his studies on human subjects found that increasing the energy content of a meal of fixed composition progressively delayed gastric emptying. A recent study on gastric emptying of low and high caloric liquid meals measured using ultrasonography in health volunteers found that gastric emptying was more rapid in the case of a low caloric meal compared to a high caloric meal (Mazzawi et al., 2019). In this experiment, the authors observed that the antral area of the stomach receded to nearly the fasting size after 30 mins following the consumption of a low caloric meal. They assumed that the receding of the antral area of the stomach was an indication of complete emptying of the gastric contents of the previously consumed low caloric meal.

In the present study, carotenoids delivered from OFSP, with gastric residence time of 44 mins, representing a low calorie dense test food, were more stable at all GE points compared to those delivered from OFSP+LIPID and OFSP+LIPID+PROTEIN test meals with higher caloric densities. The gastric residence time for OFSP+LIPID was 64 mins while that of OFSP+LIPID+PROTEIN was 82 mins. As pointed out in the earlier sections of this

experiment, the stability was more pronounced in β-cryptoxanthin than all-*trans*-β-carotene at all the points of GE possibly due to its physicochemical properties that make it stable in the presence of gastric fluids. It is likely that the longer residence time increased carotenoid exposure to the gastric acids and fluids of the gastric phase thereby affecting their stability.

Addition of 5% lipid to the OFSP led to the increased stability and nutrient delivery to the duodenum after 16 and 32 mins but progressively decreased their delivery after 48 and 64 mins. It was observed that the addition of 5% lipid to the OFSP test meal led to the formation of the oily layer on top of the chyme in the gastric phase, a feature consistent with previous findings (Marciani et al., 2007). In their study, it was observed that gastric acid caused unstable emulsions which led to the formation of the intragastric oil layer on the top of the chyme in the stomach. The intragastric oil layer resulted in the accelerated GE of the aqueous phase followed by the slow removal of the intragastric oil layer. It is possible that the lag phase created herein, could have contributed to the instability of the carotenoids as they were held up in the gastric phase all this while. Further addition of 5% (w/w) protein led to changes in the gastric behaviour such as increased gastric residence time, reduced rate of nutrient delivery and reduced digestive stability of both all-*trans*-β-carotene and β-cryptoxanthin.

This is not surprising as in this experiment, lean meat from skinless chicken pieces was used to provide the desired protein quantities in the food test samples. Previous studies that investigated the effect of protein digestion using both *in vitro* and *in vivo* approaches showed a decrease in the protein digestion rate (Bax et al., 2013b; Bax et al., 2013a). From this finding, it can be postulated that the presence of protein slowed down pepsin activity, which is a major determinant of protein digestibility, resulting in reduced gastric emptying into the duodenal phase. Increased residence time could potentially have been caused by reduced rate of proteolysis from the enzymatic action of pepsin on the added protein thereby delaying the emptying of carotenoids and other protein degradable products.

As the gastric residence time increases, the exposure of nutrients in a meal to gastric conditions tend to increase as well. The carotenoids in our test meal could have thus been exposed to the acidic gastric conditions causing their breakdown into low molecular weight compounds that could not be detected with our analytical methods. Several studies have reported how GE affects nutrient delivery to the duodenal phase (Mulet-Cabero et al., 2020a; Mulet-Cabero et al., 2017; Wang et al., 2020) and have all reported how, upon intestinal digestion, nutrients and carotenoids in this case, are absorbed and metabolised in a phased approach. In the present

study, carotenoids from OFSP, the test food with low caloric density, were delivered to the intestinal phase in very high concentrations compared to carotenoids derived from OFSP+LIPID and OFSP+LIPID+PROTEIN. However, despite the high concentration in OFSP, micellarisation efficiency which predicts relative bioaccessibility, was greater in the OFSP+LIPID test food. This is not surprising as carotenoids being lipophilic in nature, need to be solubilised in a lipid droplet before incorporation into mixed micelles (Failla et al., 2008; Chacon-Ordonez et al., 2019; Chilungo et al., 2019). It is this aqueous micellarized fraction that is now potentially available for absorption. While the OFSP+LIPID+PROTEIN test food with high caloric densities had appreciable amounts of lipid, the micellarisation efficiency in this product was quite low probably due to the reported protein-fibre interaction on the part of OFSP which could have inhibited carotenoid incorporation into the micellar fraction and therefore bioaccessibility of carotenoids probably due to the lipid protein interaction (Palafox-Carlos et al., 2011). In all the test food samples, β-cryptoxanthin was more bioaccessible than all-*trans*-β-carotene because of its hydrophobic properties. Xanthophylls are generally more bioaccessible than carotenes due to their non-polar character that facilitates their transfer from the micellar fraction (Petry and Mercadante, 2017).

In the present experiment, biofortified OFSP (*Ipomoea batatas*) was used as the test food sample for it represents an important food crop in many Asian, African and South American countries with significant VAD prevalence (Chitchumroonchokchai et al., 2017). People in these countries lack dietary diversity and therefore largely depend on calorie-rich diets such as sweet potato that inadvertently provide enough energy to live on (FAO, 2019). Unfortunately, ordinary sweet potato does not contain sufficient micronutrients needed for the mental, physical and well-being of humans (Sharma et al., 2016). Recent data suggests that consumption of biofortified OFSP has the potential to contribute to the provitamin A nutritional requirements especially in countries where this crop is a staple food (Chitchumroonchokchai et al., 2017). However, to derive optimal nutritional benefits from its consumption, there is need to consider food preparation methods that may provide the right energy-nutrient balance in the diet.

Results from the present study point to the fact that reduction in the caloric intake with adequate nutrient density in a diet to meet the recommended nutrient requirements may serve as an opportunity not just to improve nutritional status but also to mitigate challenges associated with other lifestyle factors such as obesity in individuals at risk. Furthermore, our findings are relevant as they can be used to support and validate the widely held view of how a low calorie

but nutrient dense diet can be used in improving human health and nutrition. Going forward, it is our considered view that policy pronouncements and advocacy initiatives that encourage the preparation and consumption of biofortified OFSP composite meals with moderate lipid and reduced protein for improved uptake of Pro-VITA carotenoids in order to sustain sufficient vitamin A body stores. Such may also serve as a huge motivation to the consumers of such foods as they make sound and healthier dietary choices.

## 4.5 Conclusion

It has been demonstrated, through dietary modification, how reduced caloric intake can be used to deliver enough nutrients for possible absorption and improved nutritional health. In the present study, restriction of protein intake was successfully used to demonstrate delivery of sufficient amounts of carotenoids for absorption.

The bioaccessibility of carotenoids from the OFSP test food with varying caloric densities was high with the use of the recently developed semi-dynamic gastric digestion models compared to those obtained with the static *in vitro* harmonised INFOGEST digestion. Variations in our results from the two *in vitro* digestion models is a clear testimony of the importance of standardising models if *in vivo* systems have to be accurately simulated.

The kinetics of gastric digestion from our test samples demonstrated that low calorie dense foods delivered high carotenoid contents to the duodenal phase which resulted in high micellarisation efficiencies and therefore more absorbable compounds. The amount of compounds transferred increased by 2-fold when 5% lipid was added but reduced after a further 5% addition of protein to the test food.

Although yet to be validated in human studies, our results on the relationship between caloric densities, gastric behaviour and nutrient delivery presents an opportunity for scientific and industrial exploitation on the possibility of food and dietary formulations that promote reduced caloric volume while increasing nutrient composition in meals for optimal physiological responses. This approach would be ideal for targeted populations maligned with nutritional related challenges in the design of healthier foods that could ultimately deliver nutritional benefits for a wider population.

# CHAPTER 5

# Dietary carotenoid uptake using cellular and tissue based model systems

## 5.1 Summary

The permeability of dietary carotenoids through epithelial tissue was compared using two different models that have previously been used to assess nutrient availability. All-*trans*-β-carotene and lutein prepared at equimolar concentration (10 µM) in artificial mixed micelles and chyme from orange-fleshed sweet potato (OFSP) gastrointestinal digestion were added to differentiated Caco-2 cells and murine intestinal tissue. Transepithelial transport and absorption efficiency in sample aliquots from both models were then analysed using a sensitive liquid chromatography tandem-mass spectrometry (LCMS-MS) with electrospray ($ESI^+$) set in the positive ionisation mode. Results showed that the mean uptake for all-*trans*-β-carotene in the mouse mucosal tissue was 60.2 ± 3.2% compared to 36.7 ± 2.6% in the Caco-2 cells with the mixed micelles as the test sample. Similarly, the mean uptake was higher in OFSP with 49.4 ± 4.1% following mouse tissue uptake compared to 28.9 ± 4.3% using Caco-2 cells for the same concentration. In relation to the uptake efficiency, the mean percentage uptake for all-*trans*-β-carotene from mixed micelles was 1.8-fold greater in mouse tissue compared to Caco-2 cells (35.4 ± 1.8% against 19.9 ± 2.6%). Carotenoid uptake reached saturation at concentrations above 5 µM when assessed with the mouse intestinal cells. These results demonstrate the practicality of employing physiologically relevant models simulating human intestinal absorption processes that compares well with published human *in vivo* data. The Ussing chamber model, using murine intestinal tissue, is thus a more efficient predictor of carotenoid bioavailability that can be a used to simulate human postprandial absorption *ex vivo*. The detection of dietary carotenoids in the basolateral chamber further confirms their bioavailability from test foods using *in vivo* methods.

*Keywords*: Ussing chamber, Caco-2 cells, permeability, carotenoids, transepithelial transport

## 5.2 Introduction

The permeability of carotenoids through intestinal tissue establishes a very important step towards the understanding of their bioavailability *in vivo*. Unfortunately, evaluation of their bioavailability has long been hampered by the limited knowledge of their absorption mechanisms as well as by the limitations presented by the experimental approaches involving laboratory animals and humans (Yonekura and Nagao, 2007). While human *in vivo* studies represent the gold standard to investigate bioavailability, challenges associated with food structure, complexity of behaviour during digestion and inter-individual variations in response often lead to misinterpretation of study results (Faulks and Southon, 2005; Desmarchelier and Borel, 2017; Reboul, 2019). Furthermore, human *in vivo* studies are expensive, time consuming, often invasive and have the potential for ethical implications (Chacon-Ordonez et al., 2019). Although no animal model accurately reflects human physiology, previous studies have demonstrated how animals can be essential in elucidating mechanisms by which food components can modulate metabolic pathways and translate information to human models (Schmaelzle et al., 2014; Chalvon-Demersay et al., 2017; Carvalho et al., 2018). Lately, with the use of *in vitro* cell culture systems and molecular techniques, the mechanisms of carotenoid absorption at cellular level have started to be unveiled (Desmarchelier et al., 2017; Durojaye et al., 2019; O Sullivan et al., 2009; Reboul, 2019). For instance, delivery of carotenoids across the apical membrane is believed to be facilitated by scavenger receptor class B type I (SR-BI), cluster determinant 36 (CD36), and possibly Niemann-Pick C1 like1 (NPC1L1) (Kopec and Failla, 2018). What is however not clear is whether these proteins are indeed involved in the direct transfer of carotenoids from the extracellular space to the interior or whether they are components of membrane complexes responsible for internalization of carotenoids (Reboul, 2013). O Sullivan et al. (2009) reported that carotenoids from the same food or meal have been found to interact with each other and at any stage during the absorption, metabolism and transport process. To date, mechanisms of carotenoid interaction during both cellular and tissue uptake systems when administered at equimolar concentration are yet to be elucidated.

The current widely accepted methods that employ the use of Caco-2 cells (derived from colon adenocarcinoma) to evaluate permeation after intestinal digestion have limitations on a number of factors, from epithelial cell composition to expression of enzyme and protein transporters (Desmarchelier and Borel, 2017; Bohn et al., 2018; Harrison and Kopec, 2018; Failla et al., 2019). To compensate for the short-comings associated with Caco-2 cell monolayers, the

Ussing chamber technique, an *ex vivo* approach using mouse intestinal tissue has successfully been used to evaluate nutrient absorption (Liu et al., 2019; Mulet-Cabero et al., 2020c). One of the challenges reported for dietary carotenoids is their low and variable bioavailability which may be linked to poor release from the food matrix and micellarisation, cellular uptake and transportation (Bohn, 2019). Coupled to this is the problem of effective detection and quantification of carotenoids in both food and biological samples. The most commonly used method for the identification and quantification of carotenoids utilizes HPLC with UV-vis detection (Gupta et al., 2015b) which is however lacking sensitivity to detect low concentrations. Application of LCMS-MS in this case is superior providing improved sensitivity and low detection limit for the identification and quantification of carotenoids at sub-pico gram level (van Breemen, 1995).

The aim of this chapter was to determine the fate of dietary carotenoids following simulated upper GI digestion of OFSP and exposure of the resulting chyme to differentiated Caco-2 cells and murine intestinal tissue. Apart from this, it was also in the interest of the project to characterize and compare the absorption efficiency of dietary carotenoids prepared in artificial micelles and at equimolar concentration through the use of differentiated Caco-2 cells and mouse intestinal tissues. To achieve this, the present study compared the uptake and trans-epithelial transportation of dietary carotenoids in the OFSP chyme and artificial micelles between differentiated Caco-2 cells, the most frequently used absorption prediction model, against the mouse intestinal tissue that provides a physiologically relevant system for measuring nutrient transport across epithelial tissue. Sample aliquots obtained from the apical and basolateral chambers of both models were then analysed using the LCMS-MS equipment.

## 5.3 Methodology

The permeability of the intestinal epithelium to nutrients is a key parameter in assessing systemic bioavailability (Sjögren et al., 2016) as the process determines the rate and extent of nutrient absorption. Currently, differentiated Caco-2 cells and *ex vivo* experiments using murine intestinal tissue are the methods of choice in understanding this phenomenon. Detailed procedures of initial cellular and tissue preparations are described in Section **2.4.1** and **2.4.7**.

### 5.3.1 Caco-2 cell model

Caco-2 cells have been well-established as a human intestinal model used in the investigations of nutrient bioactivity, bioaccessibility and absorption (O Sullivan et al., 2009). In this

experiment, passages 16-18 of the Caco-2 were seeded at a density of 5 x $10^4$ cells/$cm^2$ on transwell plate inserts with 0.4 μm pore size membrane and grown for 21 days to obtain a fully differentiated monolayer during which time the trans-epithelial electrical resistance (TEER) was measured twice weekly by a TEERS Voltohmmeter to ensure monolayer integrity was intact. The effect of test samples on cell viability was evaluated using the MTT assay. The preparation of the MTT assay is described in Section **2.4.5**. Cell viability was measured at 570 nm using a microplate reader and estimated using the following formula;

$$\textit{Cell Viability (\%)} = \frac{Absorbance\ of\ Sample}{Absorbance\ of\ control} \text{ X } 100 \qquad \text{Equation 5-1}$$

### 5.3.2 Ussing chamber model

The Ussing chamber is an *ex vivo* technique in which gut tissue is collected and mounted between two buffer-containing reservoirs (luminal and serosal chambers), thus permitting the study of absorption of compounds across mounted tissues (He et al., 2013). In the current study, a section of duodenum from a C57BL6 mouse was mounted in the Ussing chamber and the open-circuit trans-epithelial potential difference monitored during the entire period of incubation representing intestinal digestion. Trans-mucosal resistance was calculated using Ohm's law as an indicator of tissues integrity throughout the period of the experiment as described in Section **2.4.8.** For the analysis, an aliquot of 200 μL was taken from both the apical and basolateral compartments at 20 minute intervals for determination of carotenoid concentration and replaced with the corresponding volume of Ringer's solution containing 10 mM mannitol and glucose accordingly. Collected aliquots were stored under nitrogen gas at -80°C until further analysis.

### 5.3.3 Carotenoid extraction and analysis using the LCMS-MS

Fractions from cell culture, apical and basolateral solutions from both permeability models were extracted three times with ethanol and hexane containing 0.1% BHT following the procedure described in Section **2.4.12** while LCMS-MS analysis of the reconstituted extract was carried out using the ACQUITY UPLC I-Class PLUS system (Waters, USA) coupled to the TQ MS detector. Data acquisition and processing was achieved using MassLynx software version 4.1. Optimised MS conditions were as follows: Electrospray Ionisation (ESI) performed in the positive ion mode, Capillary 1.73 kV, cone: 41.27 V, source temperature:150°C, desolvation temperature: 300°C, Cone gas flow: 1L/hr, desolvation gas flow: 798L/hr, collision gas flow on. Chromatographic separation of Lutein, β-cryptoxanthin

and β-carotene was performed on the ACQUITY UPLC BEH 130Å C18 column (1.7 µm, 2.1 x 150 mm) Waters, USA. Reverse phase elution of analytes was performed with mobile phases of 95% methanol containing a mixture of 0.01 mM Ammonium acetate and 0.1% formic acid (A) and methyl tert-butyl ether (B). A sample volume of 1 µL was injected into the LCMS system at a flow rate of 0.360 µL/min using gradient conditions indicated in Section **2.6.5**. Carotenoids were identified by comparison of retention times and absorption spectra with pure standards. All experiments were performed in triplicate.

### 5.3.4 Statistical analysis

Values obtained from the two methods were compared using one-way ANOVA, followed by Tukey's Post-hoc test using GraphPad prism version 8.1.0 (GraphPad Software, San Diego, Ca). Values are presented as means ± standard deviation (SD) and expressed as pg/g DW or pg/mL depending on sample of analysis. Statistical significance was set at $P < 0.05$.

## 5.4 Results

### 5.4.1 Electrical parameters and tissue viability evaluation

The viability of the murine tissue mounted on the Ussing chambers was assessed on the basis of open-circuit trans-epithelial potential difference which was continuously monitored using the DC-1000 multichannel voltage clamp unit and recordings collected every 20 minutes over the 120 minute experimental period using the Spike2 8.08 software (Figure 5-1 **A**). The relative change in the TEER values was used to estimate the viability of the tissue segment over the course of digestion. The initial TEER as well as the relative change in potential difference and resistance values were recorded at 20 minute intervals to allow a period of equilibration after changing buffer solutions in the chambers. Results show that there was a slow and steady decrease in the TEER readings with a steep fall after 90 minutes on the intestinal mucosa tissue Figure 5-2A. The diffusion of Lucifer Yellow (Figure 5-3) was used to assess tissue integrity following procedures described in Section **2.4.8.** The permeability of LY on tissue system was found to be < 3%.

### 5.4.2 Caco-2 cell monolayer integrity and test of cell differentiation

The Caco-2 monolayer integrity was confirmed by recording the trans-epithelial electrical resistance measurements prior to and after the experiment. In the present experiment, the integrity of the cell monolayer was measured every third day using the epithelial voltohmmeter.

On the day of the experiment, only monolayers with TEER values above 500 Ω.cm$^2$ were used for permeability studies (Figure 5-2**B**). In order to confirm that the Caco-2 cells used in the study had fully differentiated to express both morphological and functional features of mature small intestinal enterocytes, the activity of alkaline phosphatase, a brush border enzyme, was measured. In the present experiment, the substrate, p-NPP was added to the apical compartment maintained for 3, 7, 15 and 21 days on polycarbonate filter inserts and sampled for ALP assay at the indicated time points using experimental procedures in Section **2.4.6** as shown in Figure 5-1.

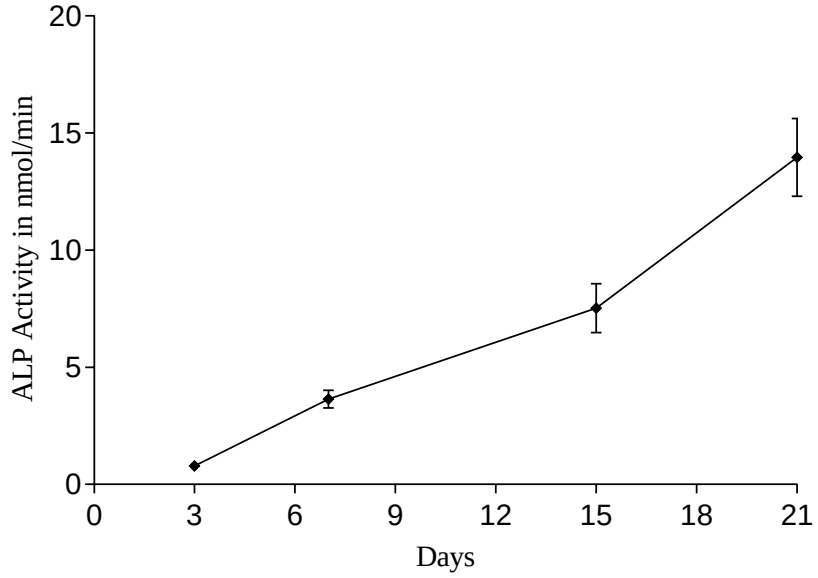



Figure 5-1: ALP activity during Caco-2 cell differentiation assayed in situ every 3$^{rd}$ day for 21 days. Data are means ± SD ($n$ = 3).

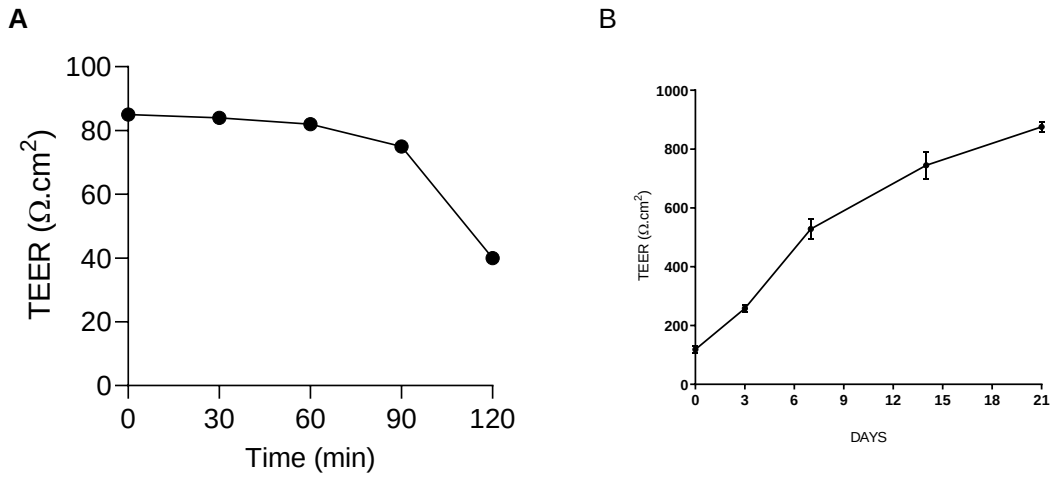



Figure 5-2: Time-dependent changes in TEER values for stripped duodenal sections in tissue (A) obtained using DC Voltage clamp and Caco-2 cells (B) obtained using a Millicel-ERS device (Millipole). Values are means ± SD ($n$ = 3).

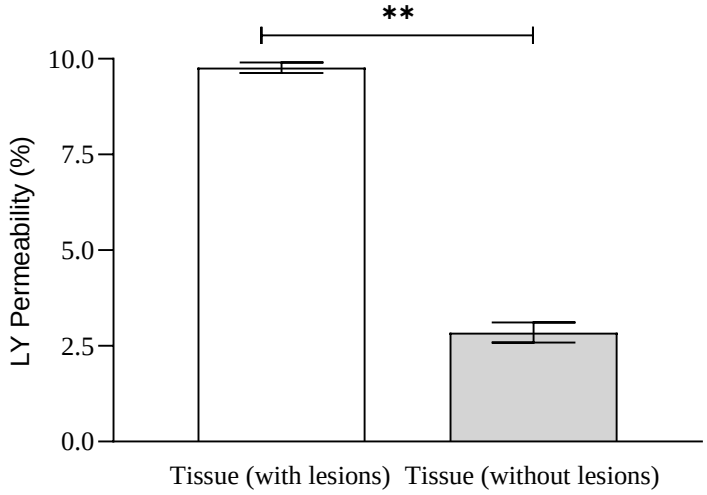



Figure 5-3: Permeability of Lucifer Yellow (LY) through mouse intestinal tissue (□ tissue with lesions) and (◙ tissue without lesions). Values are means ± SD ($n$ = 3).

### 5.4.3 Caco-2 cytotoxic assay (MTT assay)

To determine the effect of bile acids on cell viability, Caco-2 cells were pre-treated with bile acid concentrations ranging from 1.25 to 10 mM and incubated for 24 hours. Results (Figure 5-4) showed that cell viability was at an average of 87 ± 6.5 % upon the use of 1.25 mM and 10.7 ± 4.3% at 10.0 mM bile acid concentration. These results indicate that bile acid concentration at 1.25 mM did not cause significant damage to the cells within the incubation period and thus did not adversely affect cell viability. For the delivery of carotenoids to the differentiated Caco-2 cells, different concentrations of carotenoids (up to 20 µM) in artificial mixed micelles were prepared to mimic lower and higher physiological concentrations of carotenoids as described elsewhere (O'Sullivan et al., 2007; Milani et al., 2017). When cells were treated with different concentration of β-carotene and lutein, none of the lower concentrations (up to 10 µM) affected the cells in a significant way as viability was well above 70% (Figure 5-5). With a concentration of 15 µM, viability in cells treated with β-carotene significantly reduced to 65 ± 1.4% compared to lutein that had 71 ± 1.4% ($P < 0.05$). Further significant reductions were observed in the viability of cells treated with 20 µM β-carotene resulting in 50.5 ± 3.5% against 67 ± 2.8% for lutein ($P < 0.05$). Micelles without carotenoids and ordinary cells on their own were used as controls with no significant effect on the viability of the cells.

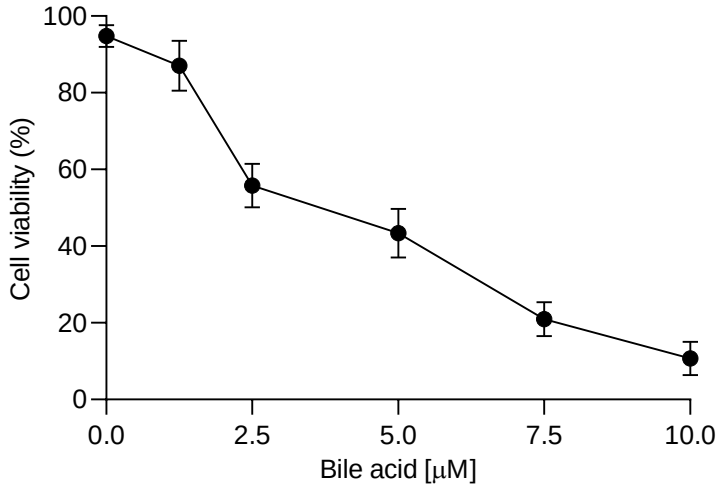



Figure 5-4: *In vitro* cytotoxicity of bile acid at different concentrations on Caco-2 cells determined by the MTT assay after exposure for 24hrs. Results are given as means normalised percentage values ± SD ($n$ = 3).

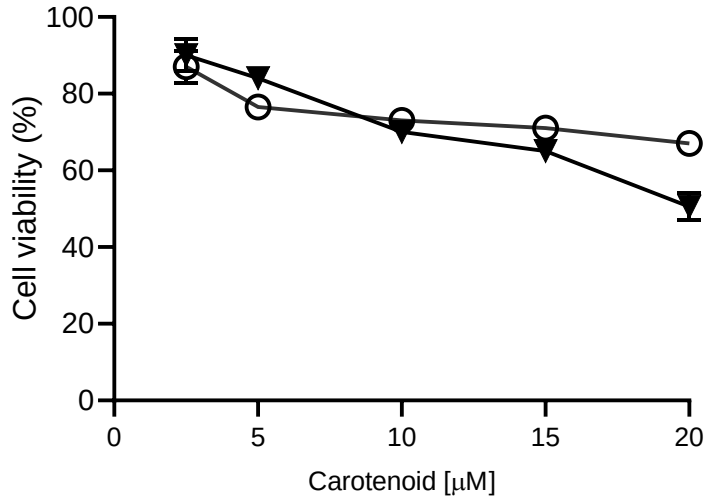



Figure 5-5: *In vitro* cytotoxicity of β-carotene (▼) and lutein (◙) at different concentrations on Caco-2 cells determined by MTT assay. Results given as mean values ± SD ($n$ = 3).

### 5.4.4 LCMS-MS Results

Carotenoids in the sample extracts were identified and quantified using the LCMS-ESI$^+$ by comparing their chromatographic retention times and full scan ($m/z$ 200 – 650) mass spectra with those of authentic standards. Using optimal LCMS-ESI$^+$ parameters (Table 5-1), mass spectra for β-carotene, lutein and cryptoxanthin were characterised by abundant molecular radical cations $[M]^{\cdot+}$. The generated ions were as follows; $[M-92]^+$ ($m/z$ 444) ion with a retention time of 5.66 minutes for β-carotene, $[M-92]^+$ ($m/z$ 460) with a retention time of 3.24

minutes for β-cryptoxanthin and $[M-92]^+$ (*m/z* 476) with a retention time of 1.99 minutes for lutein (Figure 5-6).

Table 5-1: LCMS-ESI Parameters for electrospray ionization (positive)-tandem quadrupole mass spectrometry (ESI(+)-MS/MS).

| COMPOUND | PRECURSOR ION (*m/z*) | PRODUCT ION (*m/z*) |
|---|---|---|
| Lutein | $[M]^{\cdot+}$ (568) | $[M - C_6H_6CH_3]^+$(476) (463) (551) |
| β-cryptoxanthin | $[M]^{\cdot+}$ (552) | $[M - C_6H_6CH_3]^+$(460) |
| β-carotene | $[M]^{\cdot+}$ (536) | $[M - C_6H_6CH_3]^+$(444) |

Ionisation of carotenoids in OFSP and artificial micelles to form precursor and product ions was by multiple reaction monitoring (MRM) transitions as shown in Table 5-1.

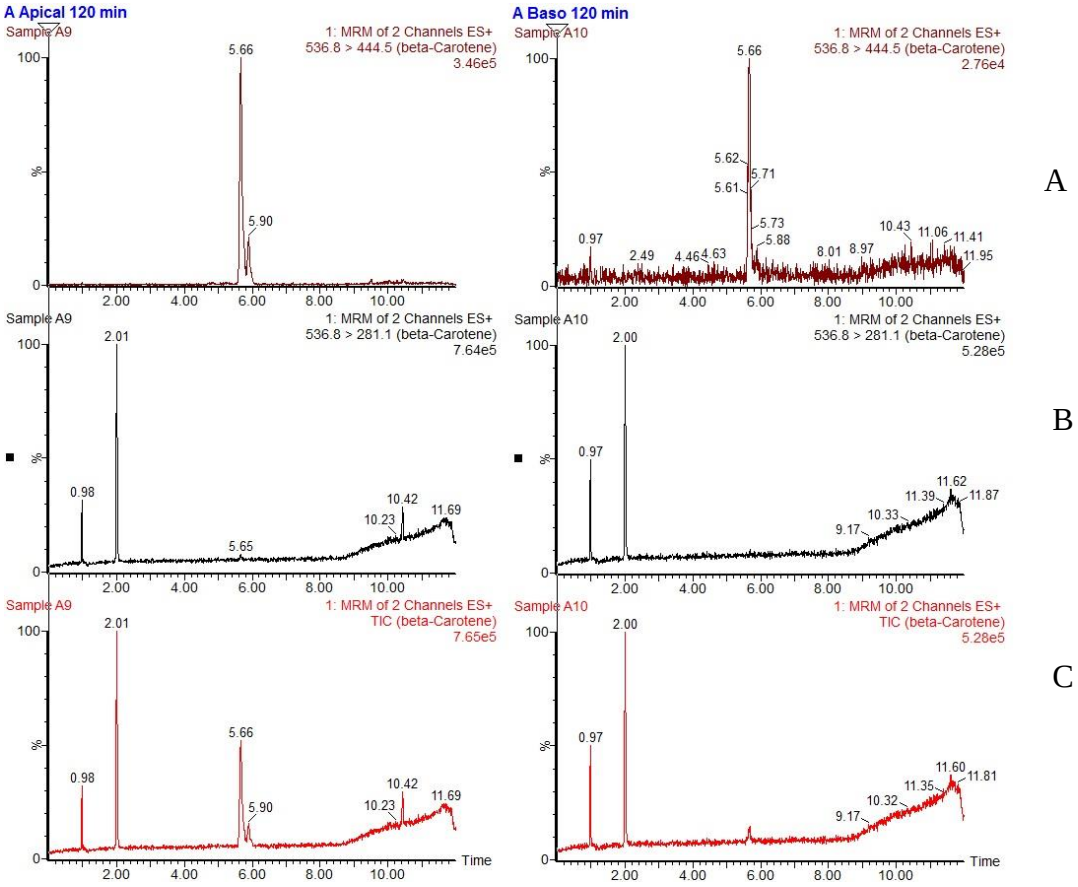



Figure 5-6: LCMS-MS chromatogram from the OFSP micellar fraction after incubation in the apical and basolateral compartment of the Ussing chambers. Results show specific SRM transitions (A and B) and TIC (C).

### 5.4.5 Caco-2 cellular uptake and transport studies

The uptake and transport of carotenoids in Caco-2 cell monolayers after 4 and 16 hours incubation is presented in Figure 5-7 **(A and B)**. Based on results from cytotoxicity assays,

artificial mixed micelles containing an equimolar concentration of 10 µM for both lutein and β-carotene and taurocholate were delivered to the differentiated Caco-2 cells. Results were then compared with delivery of micelles generated from OFSP chyme that had a two-fold dilution with DMEM to avoid cell toxicity and also maintain similar sample composition with that used in the Ussing Chambers. Carotenoid uptake by Caco-2 monolayers after 4 hours was estimated and expressed as a percentage of accumulated carotenoids in the cells. The mean cellular content of β-carotene represented 36.7 ± 2.5% in artificial micelles and 7.2 ± 0.8% in the chyme from OFSP of the amount of the total carotenoids added to the differentiated cells at the beginning of the exposure period. The pattern was similar for lutein which had 19.9 ± 2.5% from artificial micelles compared to 3.7 ± 0.6% from the OFSP chyme. Under equimolar concentrations, the cellular uptake of β-carotene exceeded ($P < 0.05$) that of lutein (Figure 5-7**A**). In the second part of the experiment, carotenoids prepared in artificial micelles were used to investigate their transportation upon incubation on Caco-2 monolayers for 16 hours. Surprisingly, no carotenoids were detected in the basolateral chamber (Figure 5-7**B**). However, at equimolar concentration of the carotenoids, there was a significant accumulation of β-carotene representing 44.5 ± 1.6% of the original concentration compared to the amount accumulated by lutein 24.2 ± 1.8% ($P < 0.05$) in the cells (Figure 5-7**B**). Furthermore, when physiological micelles generated from OFSP chyme were used to investigate carotenoid transport, very low concentrations of carotenoids were detected in the cells and again none was detected in the basolateral chamber (Figure 5-7**C**). After 16 hours incubation, the distribution of carotenoids in the cells were 9.8 ± 0.5% for β-carotene and 5.1 ± 0.6% for lutein. Despite the longer incubation time, the apical chamber still had high concentrations of carotenoids with β-carotene having 82.7 ± 2.9% while lutein had 79.4 ± 5.2% of the initial concentration.

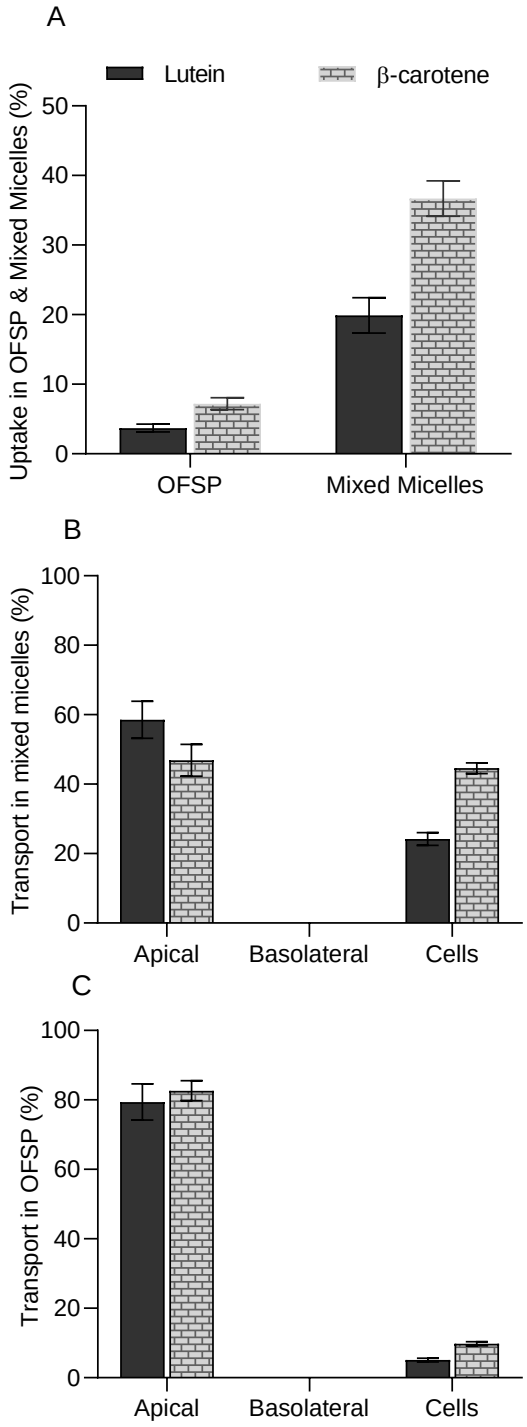



Figure 5-7: Uptake and transportation of carotenoids in OFSP and artificial micelles after different incubation times. Results show total uptake of (A) carotenoids in OFSP and artificial micelles after 4 hours of incubation, (B) transport of carotenoids in artificial micelles across differentiated Caco-2 cell monolayers following incubation of 16 hours and (C) transport of carotenoids in artificial micelles. Values are means ± SD ($n$ = 2).

### 5.4.6 Mouse tissue permeability studies

In order to determine the fate of dietary carotenoids once exposed to the intestinal mucosa, Ussing chambers containing murine intestinal mucosa had the apical chamber filled with a two-fold dilution of OFSP sample following *in vitro* digestion (Figure 5-8). Transportation and absorption of carotenoids was assessed by measuring the concentration of carotenoids in both the apical and basolateral chambers as a function of time. The carotenoids in the apical chamber represent content that was digested and therefore became accessible whereas the carotenoids in the basolateral side represents content that had been absorbed and transported across the intestinal wall and were available for physiological functions and storage. Results in Figure 5-8 state that a progressive increase in the concentration of both lutein and β-carotene carotenoids in the basolateral chamber for 90 minutes when it stabilised until the end of the 2 hrs incubation period. However, the rate and extent of these absorption processes were different between the two carotenoids. For instance, after 30 mins during incubation, 10.8% of lutein and 23.9% of β-carotene of the initial concentration had translocated to the basolateral chamber. The lutein concentration in the basolateral chamber increased to 15.2% after 60 mins incubation and 17.4% after 90 mins. In contrast, β-carotene increased 2 fold after 60 mins. After 90 mins, lutein increased to 17.4% at the end of the incubation period. Similar observations were made with β-carotene where an increase of 51.8% was recorded after 90 mins with no further increases at the end of the incubation period.

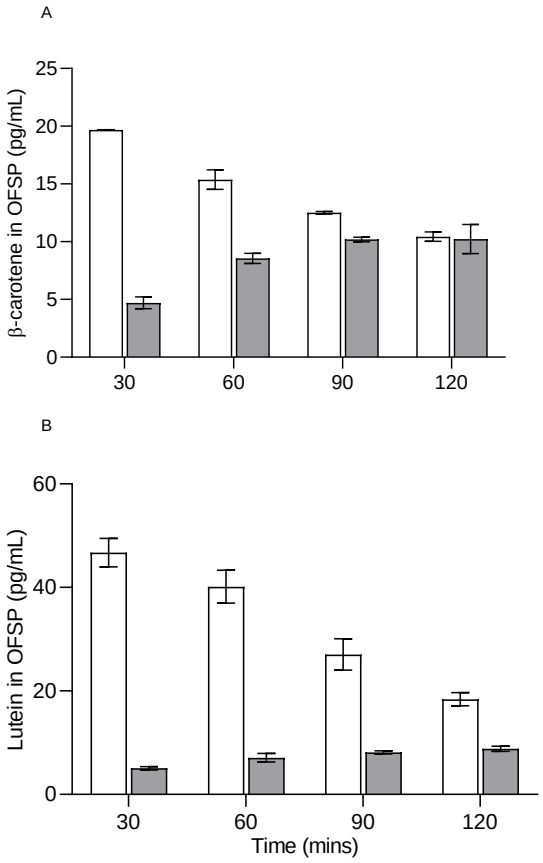



Figure 5-8: Carotenoid uptake determined by concentration change from the apical (□) to the basolateral (■) chamber in (A) β-carotene and (B) lutein in OFSP after exposure to murine intestinal mucosa. Data are means ± SD (*n*=2).

### 5.4.7 Model-based uptake efficiency

The mean uptake for all-*trans*-β-carotene in the mouse mucosal tissue was 60.2 ± 3.2% compared to 36.7 ± 2.6% in the Caco-2 cells with the mixed micelles as the test sample (Figure 5-9). Similarly, the mean uptake was higher in OFSP with 49.4 ± 4.1% following mouse tissue uptake compared to 28.9 ± 4.3% using Caco-2 cells for the same concentration. In relation to the model uptake efficiency, the mean percentage uptake for β-carotene in the mixed micelles was 1.8-fold greater with the mouse tissue than that obtained with the use of Caco-2 cells (35.4 ± 1.8% against 19.9 ± 2.6%). The trend was similar with OFSP as mean percentage values obtained indicated 31.3 ± 1.5% with the mouse mucosal tissue compared to 15.6 ± 2.0% from the Caco-2 cells. Our results further indicate that the uptake of carotenoids in both cases was higher when assessed with the tissue system compared to the Caco-2 cells.

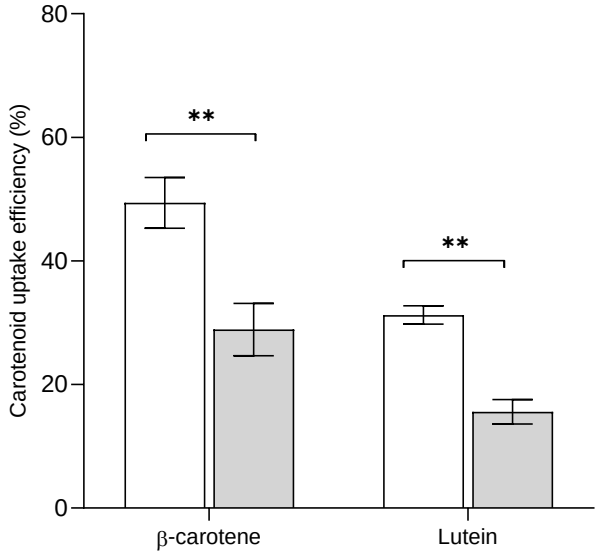



Figure 5-9: Transport efficiency of lutein and β-carotene prepared in artificial micelles at equimolar concentrations (10 µM) on mouse intestinal tissue (□) and differentiated Caco-2 cells (■). Results are presented as mean ± SD (*n*=2).

## 5.5 Discussion

The uptake and transport of carotenoids was compared using two different models that have previously been employed to assess their availability. In the present study, carotenoids prepared in artificial micelles and those generated from the OFSP chyme were successfully delivered to both differentiated Caco-2 cell monolayers and mouse intestinal tissue. *In vitro* digestion models have been used to study the bioaccessibility and thus predicting bioavailability of nutrients. However, for these models to produce reliable data, their applicability in simulating real physiological processes during intestinal digestion needs proper assessment if the prediction of bioavailability under *in vitro* conditions are to remain relevant. The most common methods are the viability and integrity of the intestinal tissue. Results of such tests are important in giving credence to the validity and interpretation of obtained data (Nejdfors et al., 2000; Sjögren et al., 2016). For example, upon removal from the animal, the *ex vivo* intestinal preparation incubated in the superfusate buffers has been reported to have limited viability lasting up to 3 hours in the Ussing chamber (Clarke, 2009). Therefore, studies using intestinal tissues need to be conducted within a defined time frame to ensure the tissue is viable in order to produce reliable data. For the present experiment, the tissue was used within three hours of collection. In the case of Caco-2 cells, no cytotoxicity was found when cells were incubated with bile salts at concentrations below 1.25 mM, indicative of no major damages to the cells

within the incubation period and therefore no adverse effect on the integrity of the Caco-2 cell monolayer on the basis of the obtained average TEER value of 600 Ω $cm^2$. These results are consistent with previous findings were TEER values above 250 Ω $cm^2$ and up to 5 mM bile salt concentrations were found to preserve tight junctions and monolayer integrity in Caco-2 cells (Antoine et al., 2015). In the current study, bile salt in a concentration of 1.25 mM were used as this has been found to be the physiological concentration during the early stages of duodenal digestion (Antoine et al., 2015). Therefore, apart from this concentration being considered physiologically relevant following meal administration, it was also found to be adequate in view of the bile salt toxic effects on the Caco-2 cell monolayer. The isolation, identification and quantification of carotenoids was conducted on the LCMS-MS with ESI in the positive ionisation mode following a procedure described elsewhere (Li et al., 2005) with modifications. Considering that carotenoids belong to a class of non-polar compounds, lacking a protonation site for ionization that enables mass spectrometric detection, our modification of the analysis method included changes in the preparation of the mobile phase by the addition of formic acid at 0.1% concentration in methanol to enhance carotenoid ionisation. Formic acid has been reported to reduce mobile phase pH and thereby decreasing signal suppression in LCMS-MS-ESI methods (Liigand et al., 2017; Cortés-Herrera et al., 2019). In the present study, the reaction of the methanol/MTBE mobile phase with 0.1% formic acid created protonated solvent clusters with the carotenoids that carried the charge during ionisation. Liigand et al. (2017) has previously reported that ionization efficiency in chromatographic analyses is dependent on the mobile phases used. In relation to the use of the HPLC, regarded as the preferred method for the separation, identification and quantification of carotenoids found in biological samples (van Breemen, 1995), here the application of the LCMS mass spectrometry with $ESI^+$ is reported for the analysis of carotenoids post-absorption in simulated physiological conditions, as it offers comparative advantage of high sensitivity and selectivity in its operations. While LCMS-MS coupled to the Atmospheric pressure chemical ionisation (APCI) mode is a popular approach for the ionisation of lipophilic compounds that include carotenoids, the use of ion electrospray was found to provide better resolution and sensitivity compared to the corona APCI with the use of methanol and MTBE. Given that carotenes lack keto-groups such as oxygen to which protons might attach (van Breemen, 1995; Lacker et al., 1999), the current method involved the use of the LCMS-MS with $ESI^+$ with the modification of the mobile phase to include the addition of 0.1% formic acid in methanol in order to carry out solution-phase oxidation as described elsewhere (Cortés-Herrera et al., 2019). Under these conditions, β-carotene yielded molecular ion $[M]^{\cdot+}$ at *m/z* 536.8; lutein at *m/z* 568.4 and β-

cryptoxanthin at *m/z* 552.4. Our results are in agreement with previous studies (Arrizabalaga-Larrañaga et al., 2019) who generated similar molecular ions with similar spectrometric conditions. Fragmentation of β-carotene gave rise to the common product ion with high intensity at m/z 444 $[M-92]^{\cdot}$, which is a typical β-carotene fragment ion formed by free-radical fragmentation from $[M]^{\cdot+}$ resulting in the loss of the toluene molecule (Lacker et al., 1999; Li et al., 2005) as well as with the formation of an additional prominent fragment ion at *m/z* 281. The fragment ion could not be identified but a previous study (Andreoli et al., 2004) reported formation of structurally significant ions during β-carotene fragmentation that included ion formation at *m/z* 281. The authors attributed the generation of this fragment ion to the cleavage on the intermediate single bonds with the retention of the charge possibly on either side of the C14-C15 carbon atoms. Product ions formed after lutein fragmentation included signals at *m/z* 476, 551 and 463. The fragmented product ion at *m/z* 476 [M-92] corresponding to the loss of toluene; *m/z* 463 [M+H-106] corresponding to the loss of two water molecules and toluene and *m/z* 551[M+H-18] corresponding to the loss of a water molecule. This finding is in agreement with previous studies that found similar fragment ions upon lutein fragmentation using different ionisation sources (de Rosso and Mercadante, 2007; Rivera et al., 2014; Cortés-Herrera et al., 2019).

### 5.5.1 Uptake and transport of carotenoids in Caco-2 cells

One of the major objectives of the present study was to investigate cellular accumulation and secretion of dietary carotenoids across differentiated Caco-2 cell monolayers. This was achieved by the use of the micellar fraction generated from OFSP chyme after simulated upper GI digestion and artificial micelles containing an equimolar concentration of β-carotene and lutein carotenoids. The use of Caco-2 cell monolayers in simulating intestinal absorption is well established and has previously been used to study the uptake and secretion of dietary carotenoids (Garrett et al., 1999; Reboul et al., 2005; O'Sullivan et al., 2007; Desmarchelier and Borel, 2017; Durojaye et al., 2019). In the present study, concentrations of carotenoids added to the cells were similar to those that have been used elsewhere to assess their cellular uptake and transport (During et al., 2002; Reboul, 2013). Results obtained in the present study showed that cellular uptake for all-*trans*-β-carotene was greater than that of lutein in both OFSP and artificial micelles in the uptake and transport models. For instance, incubation of Caco-2 cells with a combination of all-*trans*-β-carotene and lutein at equimolar concentrations of 10 µM resulted in cellular uptake of 3.67 µM and 1.99 µM, representing a percentage uptake of 36.7% for β-carotene and 19.9% for lutein, respectively. Although current cellular uptake

results were relatively high, the preferential uptake of β-carotene at the expense of lutein was similar with previous findings. O Sullivan et al. (2009) reported that supplementation of Caco-2 cells with 1 mM β-carotene or lutein resulted in cellular uptake of 12% β-carotene and 8% lutein. Similar findings have been reported by Sugawara et al. (2001) who found that the cellular uptake of β-carotene was higher than that of lutein from micelles containing 1 mM carotenoids. The preferential uptake of β-carotene is in contrast to previous studies that demonstrated preferential uptake of lutein at the expense of β-carotene (Yao et al., 2019). The probable explanation for the higher intracellular β-carotene levels could be due to its stability when formed as an emulsion compared to that formed with lutein. In support of this argument, Borel et al. (1996) found that the emulsified form of β-carotene was taken up with higher efficiency than a more polar carotenoid due to the stability of the emulsified compound. Interestingly, the present study observed that carotenoids in artificial micelles were more absorbable than those from the physiological micelles generated using OFSP. These findings are supported by previous works (Sugawara et al., 2001; Chitchumroonchokchai et al., 2004; Dhuique-Mayer et al., 2007) suggesting that the phospholipid and molecular composition of artificial micelles such as differences in particle size and surface charge may play a critical role in their interaction with the brush border surfaces of the enterocytes and therefore, absorption. This finding is supported by a human study whose aim was to assess the differences in absorption performance between β-carotene and lutein. Upon examining serum responses of single doses of β-carotene and lutein, both alone and as equimolar mixture, Kostic et al. (1995), found β-carotene to significantly reduce the serum responses for lutein to 53 - 61% of control values when taken as a combined mixture (Kostic et al., 1995; Liu et al., 2004). This therefore suggests an interactive effect that carotenoids have on each other and possibly explain the competition that exists during uptake, absorption and subsequent metabolism. A further hypothesis is that the preferential uptake of carotenoids is due to the matrix effect, taking into account localization of carotenoids in the food matrix as organelles in which carotenoids are located may determine the extent of carotenoid liberation and solubility. Failla et al. (2019) suggested that carotenoids in the food chyme may not solubilise to the same extent compared to pure compounds during digestion resulting in decreased bioavailability of the former. Regarding simulations of carotenoid secretion into the lymphatic system, the amount of carotenoids transported to the basolateral chamber correlates linearly with the amount absorbed by the Caco-2 cell monolayers regardless of the initial compound concentration at the apical side (During et al., 2002). Until recently, the long held view regarding carotenoid absorption has been that of occurring through passive diffusion (Hollander and Ruble, 1978). However,

research in the last decade has seen developments supporting the theory that several distinct proteins are involved in the facilitated transfer of carotenoids present in the micelle into enterocytes after digestion. These proteins include scavenger receptor class B type 1 (SR-B1), NPCI-like intracellular cholesterol transporter (NPCILI) and cluster of differentiation 36 (CD36) (During and Harrison, 2004; Reboul, 2013; Bohn et al., 2019; Failla et al., 2019; Reboul, 2019) which are known to be highly expressed at the apical side of the differentiated Caco-2 cell monolayers (Reboul et al., 2005; O Sullivan et al., 2009). At physiological concentrations, carotenoid absorption is carrier-facilitated and occurs by passive diffusion (Reboul, 2019; Reboul and Borel, 2011). The concentration of 10 µM used in the current study for preparation of artificial micelles was well within the acceptable physiological concentrations as previous studies have reported values of around 7 µM in human duodenal lumen for lutein (Tyssandier et al., 2003; Reboul et al., 2005) whereas an instantaneous absorption of a 5 mg dose of β-carotene, a typical amount of β-carotene found in a carotenoid-rich diet, would theoretically result in an approximate intestinal concentration of 25 µM (Borel et al., 1998; Novotny et al., 2010). In examining transport mechanisms across differentiated Caco-2 cells, carotenoid enriched micelles from both artificial and physiological preparations were incubated for 16 hours and their secretion assessed in the basolateral chambers. The present study was unable to detect basolateral secretion of carotenoids from both micelle preparations. This is in line with a previous study (Dhuique-Mayer et al., 2007) where, despite using similar methods with the present study except for differences in the membrane pores in the transwell plates, no compounds could be detected in the basolateral chamber. In contrast, some other reports have indicated carotenoid presence in the basolateral compartment of the transwell system (Aherne et al., 2010; Chitchumroonchokchai et al., 2004; During et al., 2002; Kostic et al., 1995; O'Sullivan et al., 2007; Reboul et al., 2005). Discrepancies in these findings may be related to differences in membrane pore sizes on the transwell plates and the use of taurocholate and oleic acid for the stimulation and secretion of chylomicrons into the basolateral chamber in some studies (Chitchumroonchokchai et al., 2004; O'Sullivan et al., 2007; Aherne et al., 2010). During et al. (2002) and Reboul et al. (2005) used membrane pore sizes of 3 µm and 1 µm respectively. The rationale for the use of membranes with large surface pore sizes in these studies is not entirely clear but the basis for using the 0.4 µm in the present study stems from previous studies demonstrating that transwell membranes with larger pore sizes tend to allow the migration of cells from the apical side to the basolateral side (Seeballuck et al., 2003; Seeballuck et al., 2004). Our method, similar to Reboul et al. (2005), did not add chylomicron stimulating compounds as the aim was to reproduce physiochemical conditions

in the intestinal lumen involving mixed lipid micelles containing bile salts, fatty acids, monoglycerides and phospholipids. Carotenoids being lipophilic in nature, follow the fate of lipid digestion, particularly during the process of emulsification with bile salts from the gall bladder and pancreatin from the pancreas, presumably with the correct physiological concentrations, to produce a micellar fraction which goes on to be absorbed across the epithelial cell membrane. It is therefore surprising to observe that differentiated Caco-2 cells in the indicated studies were supplemented further with taurocholate and oleic acid to enhance chylomicron secretion in contrast to what would adequately mimic the full scope and physiology of human digestion and absorption. The extent to which humans would derive maximum health and nutritional benefits from dietary carotenoids is dependent on their bioavailability. While experiments involving human subjects constitute the gold standard, whose results may provide the highest scientific evidence on the bioavailability or bioactivity of a particular compound, limitations related to the experimental design, ethical considerations, high costs in conducting the study and challenges in interpretation of results have been observed (Cilla et al., 2018). For the present study, the initial hypothesis of carotenoid bioaccessibility was tested using the static digestion model before extending the assay to the semi-dynamic digestion model owing to the complexity of the test sample. Insights gained from the dynamic digestion model were then fed back into the design of physiologically relevant screening methods to assess carotenoid permeability. During tissue uptake using the Ussing chamber, a linear increase in the absorption of both carotenoids was observed for 90 minutes before recording a plateau in the absorption curve. These results suggest that the carotenoids reach a saturation point with 51.8% of all-*trans*-β-carotene and 17.4% of lutein from the initial concentration being absorbed and transported across the intestinal wall. This is in agreement with previous human studies where plasma response after ingestion of nutritional doses of β-carotene and lutein have been reported to respond linearly before the onset of carotenoid transport saturation (During et al., 2002; Garrett et al., 1999). More recently, and although working on different compounds from test samples used in the present study, Mulet-Cabero et al. (2020c) observed lower concentrations of some amino acids in the basolateral chamber when compared to the apical section after incubation and attributed this to the reduced area of the mouse tissue in the Ussing chamber that could have led to the saturation in the tissue and therefore hampering amino acid transport. Our results further indicate that the tissue uptake for lutein was particularly low. Similar results were obtained in an experiment by Reboul et al. (2005) where the lutein uptake rate was found to be saturable under physiological conditions (30°C, 30 minutes) described by a hyperbolic equation. Using the Michaelis-Menten equation,

the authors found K value (concentration required to reach half of the maximum rate of absorption) to be 2.78 µM, reaching a saturation at approximately 5.56 µM for lutein. This value is in agreement with previous findings where measured lutein concentration in the human duodenal lumen was around 5-7 µM after a spinach-rich meal (Tyssandier et al., 2003). A human study that investigated intestinal absorption of β-carotene, lycopene and lutein found that the absorption of β-carotene maybe a saturable process considering that only about 3.5% of a 50 mg dose was able to be detected in the TAG-rich lipoprotein fraction (O'Neill and Thurnham, 1998). They concluded that less than 2 mg of β-carotene from the supplement was absorbed irrespective of the dose, confirming the β-carotene saturation process. Results from the tissue experiment in this study, which found the bioavailability of β-carotene from test meal to be 10.3% are supported by previous studies that used more accurate isotopic tracer methods to assess bioavailability of β-carotene in test meals and pure sources to be in the range of 3 and 16 % (Edwards et al., 2001; Lin et al., 2000). Furthermore, it has been demonstrated that determination of bioactive compounds in foodstuff is insufficient for the prediction of potential *in vivo* effects, as metabolites reaching the circulation may be different from the original compounds found in the food, as a result of intensive metabolism that takes place during absorption (Carbonell-Capella et al., 2014). To better understand the permeability of carotenoids, the mouse intestinal mucosa was used to investigate the prediction of percentage absorption with the Ussing chamber that facilitates more accurate prediction of percentage absorption *in vivo*. In the present study, the carotenoid absorption efficiency was 1.8 times higher when assessed by the mouse intestinal mucosa than that were obtained with the differentiated Caco-2 cell monolayers. The fact that carotenoids were not detected in the basolateral section of the transwell system of the Caco-2 cell provides some evidence on the shortcomings of the model. This finding is however not surprising as the tissue system on the Ussing chambers is considered to provide physiologically relevant conditions for measuring the transport of nutrients across various epithelial tissues (Mulet-Cabero et al., 2020c). The failure to detect carotenoids in the basolateral chamber of the Caco-2 transwell system could be as a result of exclusion of the supplementation step involving the addition of taurocholate and oleic acid to induce chylomicron secretion. Interestingly, of all the total β-carotene secreted by Caco-2 cells, 80% is associated with chylomicrons, pointing to the importance of chylomicron assembly for β-carotene secretion into the lymph *in vivo* (Harrison and Kopec, 2018). It has been reported elsewhere that under normal cell culture conditions, Caco-2 cells are unable to form and secrete chylomicrons (During et al., 2002). The high oleic acid and taurocholate has been reported to induce intracellular triglyceride synthesis and thus, facilitates

chylomicron formation (During and Harrison, 2004). Secondly, due to their hydrophobic nature, it is unlikely that carotenoids could cross the aqueous intracellular compartment without being bound to specific transport proteins. Indeed, Liver fatty acid binding protein (L-FABP), reported in the transportation of large molecules in its hydrophobic pocket, seems to be the likely candidate (Desmarchelier and Borel, 2017) for the transportation of carotenoids in physiological conditions. Unfortunately, this protein is not expressed in the Caco-2 cell monolayers and a likely reason for these carotenoids not to be secreted in the basolateral compartment.

## 5.6 Conclusion

To the best of our knowledge, this is the first study that uses biofortified test food digesta generated from a semi-dynamic digestion model that takes into account relevant physiological parameters and compares the carotenoid absorption kinetics using Caco-2 cell monolayers with a more physiologically relevant system employing mouse intestinal mucosa. The fact that carotenoids were detectable in the basolateral section of the Ussing chamber demonstrate two things: (1) that carotenoids are bioavailable under tissue system experimental conditions and (2) that, unlike the use of the Caco-2 cell model, the Ussing chamber model can be used as a more realistic approach to study the absorption and transport of carotenoids through mouse intestinal tissue simulating human intestinal absorption processes. In this study, carotenoids were detectable in the basolateral chamber of the Ussing chamber and not in the transwell system of the differentiated Caco-2 cells. Secondly, based on the absorption efficiency, there's a preferential uptake of all-*trans*-β-carotene at the expense of lutein even when these compounds are prepared at equimolar concentrations. These findings add to the increasing body of knowledge demonstrating the practicality of employing the Ussing chamber model in obtaining carotenoid permeability data that compare very well with published human *in vivo* data. Thus, the Ussing chamber model is suitable to represent carotenoid bioavailability in humans.

# CHAPTER 6

# General discussion, future studies and conclusion

## 6.1 Research justification and novelty

There has been growing interest in the study of dietary carotenoids because of their perceived roles in preventing diseases associated with both metabolic and degenerative conditions. Some carotenoids are also known to be precursors of retinol, an active form of vitamin A. In their native form, carotenoids exist as colourful lipophilic pigments in plants, fungi, bacteria, algae and are also present in many fruits and vegetables (Milani et al., 2017). Despite their important health and nutritional benefits, humans cannot synthesize carotenoids *de novo* and can therefore only obtain them through dietary intake as preformed or provitamin A compounds. With more than 190 million pre-school children and over 19 million pregnant women being at risk of VAD, defined by serum retinol concentration < 0.7 μM (Ortiz et al., 2018), strategies are needed to counteract the deficiency. Strategies that are used include supplementation with high dose vitamin A, food fortification, increasing dietary diversity and biofortification of staple foods. However, supplementation efforts are not sustainable as they are mostly donor driven while fortification efforts have been reported suffer from regulatory and control lapses. A recent study indicated that fortification of food products with preformed retinol that have populationwide coverage could lead to hypertvitaminosis A in the consumers (Sheftel et al., 2018). The basic assumption in the dietary diversity and biofortification strategies is that continued consumption of foods rich in provitamin A carotenoids may improve the health and nutritional status of consumers in the long run. However, the impact of consuming provitamin A rich foods on improving vitamin A status is affected by not only efficiency of conversion but also other factors that include carotenoid species, processing methods and other post-harvest handling methods like storage conditions (Burt et al., 2010; Chilungo et al., 2019; Trono, 2019).

Numerous studies have been conducted that have assessed the effect of processing on the content, retention and stability of carotenoids in fruits and vegetables (Bengtsson et al., 2008; Carvalho et al., 2014; Dias et al., 2014; Berni et al., 2015; De Moura et al., 2015; Diaz-Gomez et al., 2017; Cilla et al., 2018; Chilungo et al., 2019) producing varying and in some cases, conflicting results. Apart from the commonly available seasonal fruits, the consumption of

other exotic fruits in resource-poor settings is generally low (Okop et al., 2019) as these fruits are priced beyond the economic reach of most households. Thus, the only other available dietary source of carotenoids comes from vegetables and tubers normally consumed as part of the staple food. Furthermore, and unlike in certain parts of the world where most vegetables are consumed raw and on their own, preparation and consumption of vegetables in most developing countries come in a plethora of combinations and served as composite meals. This brings into question the effect such interactions of meal ingredients would have on the eventual release and stability of carotenoids in relation to their initial concentration in the original and unprocessed matrix. Secondly, food processing has been reported to be one of the major determinants of nutrient bioavailability as it can have both positive and negative effects of increasing or decreasing the bioaccessibility of nutrients and bioactive compounds (Cilla et al., 2018). Since bioaccessibility entails release from the food matrix during digestion, some *in vitro* digestion models have been proposed mainly on the premise of simulating the transit through the human GI tract in an effort to reproduce the physiological conditions of gastric and intestinal digestion. The two common models used for the bioaccessibility of carotenoids include the static digestion model on one hand, characterized by chemical digestion and the semi-dynamic model on the other that takes into account kinetic aspects of physical and biochemical processing in the GI tract. The static model has been found to be good for endpoint measurements while the semi-dynamic digestion model, that more closely mimics *in vivo* conditions (Amorim-Carrilho et al., 2014) is capable of generating kinetic data. The simulation of muscle contraction in the semi-dynamic model has been reported to generate shear forces and fluid motions that not only enhances the breakdown of food but also facilitates chemical digestion, absorption and transport (Fernández-García et al., 2012). According to recent data suggesting that meal composition and food structure has an impact on nutrient absorption and digestion (Garcia-Campayo et al., 2018; Mulet-Cabero et al., 2019), the use of the static digestion model may fall short of adequately simulating human digestion and thus impacting nutrient absorption. Mulet-Cabero et al. (2017) observed that food structure, particularly meal composition, size and caloric content had the ability to alter gastric behaviour and therefore affect nutrient delivery and absorption in the small intestines. To the best of our knowledge, data on the kinetics of carotenoid delivery from the simulated gastric phase into the small intestine using a test meal prepared with varying degrees of calories has not been previously investigated and reported.

The next stage after the delivery of carotenoids into the small intestines from the gastric phase involves their absorption, uptake and transportation into the systemic circulation. It is at this stage that permeability of these compounds through the intestinal tissue constitutes a critical stage in defining bioavailability *in vivo*. Several authors have examined the absorption of carotenoids either in their individual pure forms or as a mixture of carotenoids (O'Neill and Thurnham, 1998; Yonekura and Nagao, 2007; Reboul and Borel, 2011; Ortiz et al., 2018; Reboul, 2019). However, these investigations have been hampered by factors such as limited understanding of carotenoid absorption mechanisms, lack of consensus on the intestinal absorption efficiency, mechanisms of enzymatic cleavage into retinol and above all, limitations presented by experimental approaches involving *in vitro* and *in vivo* studies. Currently, the Caco-2 cell lines have been accepted and widely used to predict intestinal absorption due to their ability to model human absorption characteristics (Osakwe, 2016). The major challenge is that Caco-2 cells are composed of a single layer and the absence of other cell types and intestinal mucus means that they do not represent the natural physiological environment critical during *in vivo* intestinal digestion (Lea, 2015; Osakwe, 2016). Given these shortcomings with the Caco-2 cells, the use of a tissue system may be a better alternative for permeability studies. Apart from absorptive cells, the intestinal epithelium is composed of goblet cells, endocrine cells and the M cells has been found to give a better representation of the various processes involved in the *in vivo* situation (Westerhout et al., 2015). Despite these strong salient features of the intestinal epithelium, no study has investigated the transportation of carotenoids from a test meal across the intestinal epithelium and later on compared its transport efficiency with the standard Caco-2 cellular model. Since the promotion of the consumption of biofortified foods as a strategy to mitigate VAD in the afflicted areas is gaining momentum, it remains to be seen how enhanced concentrations of different carotenoids in these foods interact and whether indeed absorption of one carotenoid might not affect the other. The synergistic effects of carotenoids would need to be clearly understood by plant breeders in their efforts to set targets to meet the population recommended dietary intake.

This study was therefore undertaken to investigate the content, retention and stability of carotenoids following different processing and meal preparation methods in butternut squash (BNSQ), biofortified maize meal and OFSP varieties. The selection of these food items was done on the basis that they constitute excellent sources of provitamin A carotenoids in the human diet and recently, biofortified maize and OFSP have been introduced as a food based strategy in reducing the occurrence of VAD. Upon analysis and discovery of high carotenoid

content in the OFSP variety, the present project further assessed the kinetics and the impact of food composition on carotenoid delivery from the simulated gastric phase into the small intestines, particularly on the effect of caloric density on gastric digestion and bioaccessibility. Finally, the study compared the uptake and trans-epithelial transportation of dietary carotenoids in the OFSP chyme and artificial micelles between the differentiated Caco-2 cells and the mouse intestinal tissue that provides a physiologically relevant system for measuring nutrient transport across epithelial tissue.

## 6.2 Characterisation and content of carotenoids in processed biofortified foods

Ordinary BNSQ and OFSP samples were initially used to optimise HPLC analysis, but results were compared as well carotenoid with biofortified food samples. The first achievement in the present study was that carotenoids in different genotypes of raw OFSP were successfully characterised and results showed high levels of *all-trans*-β-carotene, a provitamin A carotenoid, ranging from 60.1 to 80.6 μg/g DW. *Kakamega* genotype had the highest concentration (80.6 ± 3.10 μg/g DW). In the present study, boiling and baking processing of biofortified OFSP varieties led to significant reductions in the concentration levels of *all-trans*-β-carotene (71.2 ± 1.23 μg/g DW) and zeaxanthin with increases in the β-cryptoxanthin and β-carotene isomers in all the varieties. In relation to boiling, baking resulted in losses of up to 21.5% vs 11.7% for *Kakamega*. Similarly, the retention of Pro-VITA was high in boiled *Kakamega* (91.4%) compared to its corresponding baked product (83.6%). This finding is encouraging considering that the preferred method of preparation of OFSP has always been boiling particularly in rural areas of sub-Saharan Africa where it is served during lunch or dinner as boiled or a mixed dish and served with cowpeas, beans, coconut milk and/or dark green leaves (Low et al., 2017). The observed high Pro-VITA retention in almost all the varieties of OFSP is another positive score that is likely to improve and maintain total vitamin A body stores in regular consumers of biofortified OFSP. According to the US Institute of Medicine (IOM, 2001), the derived conversion factors for estimating retinol activity equivalent (RAE) obtained from provitamin A carotenoids indicate that 12 μg of *all-trans*-β-carotene is equivalent to 1 μg RAE. Therefore, the vitamin A value calculated in RAE of the OFSP in *Kakamega*, the cultivar with the highest concentration of *all-trans*-β-carotene, would range from 500 to 672 μg RAE/ 100 g. Given that the average Pro-VITA retention was found to be 91.4% after boiling, the *Kakamega* OFSP genotype would thus provide between 458 – 614 μg

per100 g ready to eat portion. Considering also that an average portion size for a child is about 125 g (van Jaarsveld et al., 2005), the *all-trans*-β-carotene concentration even at the indicated retention percentage would be more than enough to meet the WHO recommended vitamin A intake of 400 µg RE for children under the age of five (WHO, 2004). These figures, impressive as they may appear, would still need to be validated with efficacy studies since β-carotene conversion to vitamin A is dependent on other factors that include vitamin A and health status (van Jaarsveld et al., 2005).

In the case of biofortified maize, the study revealed that Pro-VITA carotenoids levels were low and ranged from 2.23 to 5.10 µg/g DW among all the genotypes (Table 3-1). However, when biofortified maize meal was processed into *Nshima meal*, as a normal culinary practice, there was a slight increase in the content resulting in the higher retention of Pro-VITA in *Nshima meal* probably due improved matrix disruption resulting from heat exposure. The fact that Pro-VITA retention improved in the *Nshima meal* as opposed to *Nshima* provides a window of opportunity on how traditional cooking methods could be optimised to derive maximum benefits from consuming Pro-VITA biofortified food products and a ray of hope for the biofortification program implementers in different countries. The current study investigated how processing affected carotenoid content in biofortified maize when foods were prepared as composite meals. Traditionally, maize meal would be consumed with an accompanying dish called relish that may be beef stew or boiled vegetable in cooking oil. The vegetable oil in this case provides the partitioning phase for carotenoid solubilisation during subsequent extraction and analysis. In the previous studies (Mugode et al., 2014; Pillay et al., 2014), analysis of carotenoids was done on Nshima alone, which in the actual context, would not be consumed as indicated and thus providing insufficient information on the potential impact of consuming biofortified foods.

Other achievements in the investigation include important information on the variations in the content of carotenoids in the biofortified food samples. For instance, three of the four varieties of maize had higher concentrations of lutein and zeaxanthin, the non-provitamin A carotenoids, than the provitamin A carotenoids. It is not yet clear whether these varieties are already on the market and are all meant to improve vitamin A status in the selected population. Assuming such foods are targeted at mitigating the problems associated with VAD, there is a likelihood that the anticipated impact of consuming these foods would not achieve the intended objective at the same rate considering the fact that carotenoids at different concentrations in food samples

are absorbed differently, an aspect addressed in **Chapter 5**. This probably explains why a recent study (Palmer et al., 2016), in a cluster-randomised trial found that provitamin A biofortified maize was only able to increase serum carotenoid levels but not retinol in marginally nourished children. Secondly, carotenoids are known to be very unstable compounds and prone to degradation at high temperatures (Che et al., 2016), however, the reality on the ground is that maize meal products remain acceptable for human consumption up to six months after grinding and in some cases, a year after storage (Ortiz et al., 2018). Information on the stability of carotenoids stored under such conditions at room temperature is limited and should be investigated. In the current study, the investigation was centred on the effects of storage on *Nshima meal,* considering that long-term storage is a normal practice of consumer's domestic behaviour. The losses of up to 50% in *all-trans*-β-carotene after 15-day storage at -20°C is suggestive that consumers may not derive the desired benefits when meals are prepared and stored for longer periods at -20°C. Consumers would need to be encouraged through nutrition and health education programmes on the advantages that come with consuming meals with short periods of preparation and avoid longer storage conditions of biofortified foods.

## 6.3 Bioaccessibility of the OFSP test meal

The second part of the present investigation was centred on the impact of food composition on carotenoid bioaccessibility with special interest on how caloric density modulates carotenoid digestion and its subsequent bioaccessibility. The main achievements from investigations conducted in **Chapter 4** were as follows:

Firstly, using nutrient composition data described in Table 4-2, energy content and gastric residence time of the test food was calculated on the basis of the Atwater factors established. Secondly, considering the fact that gastric digestion is never static, the present study employed the use of a semi-dynamic digestion model that includes crucial kinetic parameters such as gradual acidification, fluid and enzyme secretions and emptying that simulates an upper gastrointestinal tract of an adult. This is a crucial aspect to consider during meal preparation and food design as different constituents in food composition may impact on the stability and absorption meant to improve the health and nutritional status of the target population. Thirdly, on comparing the effect of different digestion models, findings in the present study revealed that the semi-dynamic model produced better recoveries in comparison to the static digestion

models. The fraction of carotenoids released is an important step in understanding the quantity of the bioactive compound that will eventually be available for absorption. Therefore, the use of digestion models that closely simulates physiological conditions should at all times be considered if the prediction of bioavailability under *in vitro* conditions are to remain relevant. For instance, in the present study, the recovery of *all-trans*-β-carotene in OFSP after intestinal digestion was 66.1 ± 1.1% on the semi-dynamic model and 58.3 ± 2.4% when analysed using the static digestion model. Similarly, the recovery of β-cryptoxanthin in the same test food was 73.0 ± 2.6% with the semi-dynamic digestion model compared to 63.9 ± 3.8% recovered with the use of the static digestion model. Previous studies have reported low and variable carotenoid bioaccessibility results (Liang et al., 2013; Reboul, 2013; Harrison and Kopec, 2018). The low recoveries have implications as their low concentrations tend to equally present challenges with detection limits which may require specialised and hard to find equipment in trying to predict compound bioavailability. The fourth and cardinal point is that, the present study was able to demonstrate, through dietary modification, how reduced caloric intake can be used to deliver sufficient amounts of nutrients for possible absorption and improved nutritional health. Because of lack of comparative literature, it was important to conduct in-depth investigations on the kinetics of carotenoid digestion in OFSP test foods with varying caloric densities. To achieve this, test foods with the following formulations were used: (i) OFSP test food alone (ii) OFSP test food with lipid (iii) OFSP test food with lipid and protein that was equalised by meal volume. Results showed that OFSP test food with low calories presented more stable *all-trans*-β-carotene carotenoids with reduced gastric residence time. In this case, the recovery of *all-trans*-β-carotene was at an average of 94.0% after 11 minutes compared to 80.5% and 72.0% for the OFSP+LIPID and OFSP+LIPID+PROTEIN after 16 and 20.5 minutes respectively. This highlights that calorie-dense test foods take longer to be emptied from the gastric chamber, the formed coalescence is less stable and contribute to reduced carotenoid bioaccessibility at the end of GI digestion. In order to maximise on the benefits of carotenoids from a biofortified meal, a balance will have to be struck during the design and preparation of foods for specific populations so that high concentrations of carotenoids can effectively be delivered and absorbed during GI digestion. Considering the postulated impact biofortification may have on the vitamin A status of humans, other food crops are having their nutrients enhanced to improve the nutritional status of their consumers. For instance, chickpea, an important source of nutrition and economic livelihood in developing countries (Sivasakthi et al., 2019), is being introduced in the semi-arid tropical regions as a cultivar with enhanced climate resilience and enhanced provitaminogenic carotenoids.

Analysis of the carotenoid content in the improved chickpea stay-green product was done during HPLC method optimisation as indicated in section 2.5.4. To derive maximum benefits, nutrition and health educators would need to provide education on the importance of consuming meals prepared from biofortified foods and under conventional or traditional methods, to sustain both adequate body stores of carotenoids and satiety levels.

## 6.4 Uptake and trans-epithelial transport of dietary carotenoids

The final achievement in **Chapter 5** of our investigations was a demonstration of how physiologically relevant models can be used to accurately simulate *in vivo* human studies in predicting bioavailability. Following GI digestion of OFSP in the semi-dynamic model as described in **Chapter 4**, the resulting chyme was exposed to differentiated Caco-2 cells and murine intestinal tissue. The aim was to characterize and compare the absorption efficiency of dietary carotenoids generated from the OFSP chyme and carotenoids prepared at equimolar concentration in artificial micelles and through the use of differentiated cultures of Caco-2 cells and mouse intestinal tissues.

Initially, carotenoids prepared in artificial micelles were incubated at 37°C on ordinary 6-well plates for the purposes of testing cellular accumulation of carotenoids prepared at equimolar concentration for 4 hours. The choice of incubation time was made based on previous studies (Reboul et al., 2005) whose objective was to measure maximum absorption rate of carotenoids that would provide sufficient amounts to enable adequate measurements. Secondly, it is also assumed that transit time for food in the small intestines does not take longer than 4 hours after digestion (Minekus et al., 2014). Therefore, incubation of carotenoids for 4 hours was within the general digestion transit time. For transport studies, carotenoids were incubated on transwell plates for a period of 16 hours with the incubation time being based on the time it takes for chylomicrons to be assembled and secreted into the lymphatic system (During et al., 2002). Ordinarily, carotenoid concentrations are determined by HPLC using the reverse-phase C30 column with capabilities to differentiate structurally similar compounds. However, this instrument could not be used in the present study because the concentrations of carotenoids after intestinal digestion in the test samples were low and could only be detected on the LCMS-MS with ESI in positive ionisation mode. Under modified and optimised conditions, mass spectra obtained for *all-trans-β*-carotene and lutein were characterised by molecular $[M]^{\bullet+}$ ions. While these two carotenoids lack a keto-groups that facilitate protonation during electrospray

ionisation, previous studies (Li et al., 2005) have demonstrated that modification of constituents in the mobile phase with a pH additive such as formic acid at a concentration of 0.1%, (v/v) was able to enhance ionisation efficiency of analytes including carotenoids. Other studies have attributed the generation of $[M]^{\bullet+}$ to electrochemical oxidation (Blades et al., 1991). The general understanding is that during the process of ionisation, the electrophoretic charging and field ionization acts as a electrolytic cell at the metal-liquid interphase of the electrospray capillary resulting in the generation of the $[M]^{\bullet+}$ for non-keto group containing carotenoids. Therefore, with these optimised conditions, and consistent with ESI(+)-MS spectra for product (daughter) ion formation, fragmentation of *all-trans-β*-carotene gave rise to product ions with high intensity at *m/z* 444. Similarly, fragmentation of lutein gave rise to product ions at *m/z* 476, 463 and 551 (Table 5-1).

Results of our uptake experiments showed that supplementation of Caco-2 cells with a combination of all-*trans*-β-carotene and lutein at equimolar concentrations of 10 µM resulted in cellular uptake of 3.67 µM, representing a percentage uptake of 36.7% for *all-trans*-β-carotene and 1.99 µM and a percentage uptake of 19.9% for lutein. As indicated in Chapter 5, it is possible that the preferential uptake of *all-trans-β*-carotene was as a result of the stable emulsions formed by β-carotene micelle fractions. During et al. (2002) reported a preferential accumulation in plasma and in postprandial lipoproteins of *all-trans*-β-carotene against other carotenoids in humans. Like most *in vivo* studies, the indicated study could not provide information on the mechanisms of the selective transport of *all-trans*-β-carotene. For this reason, the authors concluded that there was a possibility of a specific intracellular mechanism leading to the preferential incorporation into chylomicron of *all-trans*-β-carotene against other carotenoids. In the present study, **Chapter 3** revealed that 4 out of 5 genotypes of maize had higher concentrations of non-provitamin A carotenoids (lutein and zeaxanthin) compared to the provitamin A carotenoids (*all-trans*-β-carotene and β-cryptoxanthin). It may be interesting to model uptake and transport studies that investigate the effect of having one carotenoid in higher concentration against the other. Previous studies have revealed that β-carotene uptake was significantly impaired when Caco-2 cells were treated with a dispersion of lutein:β-carotene in the ratio of 4:1 (O Sullivan et al., 2009). Results of such studies may provide evidence on the need to modulate carotenoid concentration levels during biofortification breeding programs to achieve maximum benefits in targeted populations.

## 6.5 Outcome of carotenoid transport on Caco-2 cell models

Regarding simulations of carotenoid secretion into the lymphatic system, carotenoid enriched micelles from both artificial and physiological preparations were incubated for 16 hours and their secretion assessed in the basolateral chambers of the Caco-2 cells. Similar experiments were conducted on mouse intestinal tissue except in the latter, incubation was restricted to 2 hours. While carotenoids were not detected in the basolateral chamber of the transwell plates using Caco-2 cells, *all-trans*-β-carotene and lutein carotenoids were detected in the basolateral section of the mouse intestinal tissue. Previous studies were able to detect carotenoids upon stimulation of the cells in the apical section of the transwell system with taurocholate and oleic acid for the secretion of chylomicrons into the basolateral chamber (During et al., 2002; O'Sullivan et al., 2007; Aherne et al., 2010). Given that cellular studies investigating uptake and secretion of nutrients are meant to mimic human *in vivo* postprandial state, the supplementation of cells with chylomicron secreting compounds as indicated in the previous studies does not make much sense considering that these compounds were presumably already secreted during mixed micelle formation. In trying to simulate human *in vivo* intestinal absorption of carotenoids, it is only logical that attempts are made to reproduce physiological conditions of the intestinal lumen if data generated from *in vitro* studies is to be compared accurately with what obtains *in vivo*.

## 6.6 Outcome of carotenoid transport on mouse intestinal tissue

When an equimolar concentration of carotenoids was used to investigate carotenoid transport on mouse intestinal tissue, an increase in the absorption of both carotenoids for 90 minutes before recording a plateau in the absorption curve was observed. These results are suggestive of the compounds reaching the saturation point during the course of intestinal absorption. This result is in agreement with previous human studies where plasma response after ingestion of nutritional doses of β-carotene and lutein have been reported to respond linearly before the onset of carotenoid transport saturation. According to During et al. (2002), the uptake and secretion of carotenoids is a curvilinear, time-dependent, saturable and concentration-dependent process. While this statement summarizes observations made in the current study and is in agreement with a human study that made similar observations (O'Neill and Thurnham, 1998), further studies employing the use of different carotenoid concentration curves would need to be performed to validate these findings.

## 6.7 Outcome on the use of different trans-epithelial permeability models

To better understand the absorption mechanisms of carotenoids, mouse intestinal tissue was used to investigate the prediction of absorption and compared to Caco-2 cells. Results revealed that carotenoid absorption efficiency was comparatively higher with the mouse intestinal mucosa compared to the differentiated Caco-2 cell monolayers. While both models are mere predictors of bioavailability, it is important to bear in mind that determination of content of bioactive compounds in the food matrix is not enough evidence for the prediction of potential *in vivo* effects. Therefore, the use of animal models to accurately mimic human physiological conditions has been a long standing challenge. So far, only Mongolian gerbils have been found to metabolise α- and β-carotene similar to man (Davis et al., 2008). Unfortunately, animal sacrifice is needed to successfully conduct experiments on the Ussing chambers. Use of Caco-2 cells may need to be optimised for a better understanding of permeability and absorption studies. Indeed, absorption is a complex process that sometimes involves different metabolic pathways. While certain compounds may be absorbed as a whole, metabolites reaching the systemic system after digestion may be different from the original compounds found in the chyme before absorption (Capuano, 2017). This is particularly true for most provitamin A carotenoids that undergo enzymatic cleavage during digestion to produce retinol and other carotenoid metabolic products.

## 6.8 Future work

- Future research should focus on validating our findings with carefully controlled human studies using isotopic tracer methods on the aspects of how caloric density of composite meals modulates stability, digestion and bioavailability of dietary carotenoids. This is important as data generated will be based on the locally prepared composite meals and can be used by policy implementers to design locally accepted meals in the targeted populations.
- Using *in vitro* permeability assays, the isotopic tracer methods should be followed up to understand the bioaccessibility and metabolic pathways of dietary carotenoids. Information obtained from this study will help researchers understand the dynamics and factors responsible for low bioavailability of carotenoids following compound analysis at different stages of digestion.

- Pigs' intestinal tissue should be considered to assess permeability studies considering their physiological and immunological similarities to humans.
- Particle size and charge distribution of individual carotenoids prepared in artificial micelles should be analysed as these parameters have been reported to play a critical role in absorption upon their interaction with brush border surfaces of the enterocytes. Data generated from this study may help explain the preferential uptake of certain carotenoids at the expense of the other.

## 6.9 Conclusion

For carotenoids to exert their nutritional effects in the human body, they need to undergo enzymatic cleavage and converted to retinol. However, certain determinants including host-related (health, nutritional status and genetic variations in BCO1 and SNPs) and food-related (matrix, composition and meal preparation) have been cited as limiting factors (Graßmann et al., 2020). In the present study, analysis of food samples revealed that food processing and preparation techniques are important factors that ultimately reflect changes in the content and retention of the carotenoids to be consumed, absorbed and used for physiological functions. For intance, boiling instead of baking was found to be a better processing technique to increase the retention of Pro-VITA carotenoids. Similarly, the preparation of composite *Nshima meal* remarkably improved retention of the Pro-VITA carotenoids. Understanding the best food processing and preparation methods can therefore be used to help consumers derive maximum benefits from such practices. These traditional processing methods have the advantage of being simple and to fit into the ordinary meal preparation practices.

The present study demonstrated that, restriction of protein intake and moderate use of lipid can be used to deliver sufficient amounts of carotenoids for absorption.

Finally, despite its low throughput and technical expertise required in conducting the experiment, results obtained from the current study were able to demonstrate that the Ussing chamber model is a more efficient predictor of carotenoid bioavailability that can be a used to simulate human postprandial absorption *ex vivo*. This work shows that modulating the composition of lipids and proteins in a biofortified OFSP composite meal is a critical step in the delivery of dietary carotenoids for efficient intestinal absorption and subsequent availability for the maintenance of adequate vitamin A body stores in humans.

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