POST HARVEST STORAGE OF BIOFORTIFIED MAIZE IN PURDUE IMPROVED CROP STORAGE (PICS) BAGS AND EFFECT ON SUBSEQUENT FLOUR RHEOLOGY AND CAROTENOID BIOACCESSIBILITY

by

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To my lovely and beautiful wife, Beatrice; and two daughters, Trinity and Michelle.

To my late parents

To my uncle

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ACRE	Agronomy Center for Research and Education
BC	all -trans-β-carotene
BCO1	β-carotene-15, 15'–oxygenase
BCO2	β-carotene-9, 10' –oxygenase
B-crypt	β-cryptoxanthin
cP	Centipoise
DAD	Diode Array Detector
DTT	Dithiothreitol
DW	Dry Weight
EAR	Estimated Average daily Requirement
FT	Fourier Transformed
HDPE	High Density Polyethylene
HPLC	High-performance Liquid Chromatography
IOM	Institute of Medicine
LC	Liquid Chromatography
Lut	all -trans-Lutein
MTBE	Methyl ter-butyl ether
OPV	Open Pollinated Variety
PICS	Purdue Improved Crop Storage
PTFE	Polytetrafluoroethylene
pVACs	Provitamin A Carotenoids
RAE	Retinol Activity Equivalence
RH	Relative Humidity
RVA	Rapid Visco Analyzer
SAS	Statistical Analysis System
UPLC-MS	Ultra Perfomance Liquid Chromatography-Mass Spectroscopy
VA	Vitamin A
VAD	Vitamin A Deficiency
WHO	World Health Organization
Zea	all -trans-Zeaxanthin

ABSTRACT

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Title: Post Harvest Storage of Biofortified Maize in Purdue Improved Crop Storage (PICS) Bags and Effect on Subsequent Flour Rheology and Carotenoid Bioaccessibility
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Successful adoption of biofortified orange maize in developing countries requires careful consideration of factors across the chain from farm to fork. This includes consideration of post-harvest storage conditions optimal for the retention of both proviatamin A carotenoids and cooking quality critical to consumers. In these considerations, identification of economical storage methods is critical considering the limitations within specific countries that biofortified maize is being disseminated. To address these points, this dissertation research focused on evaluation of the utility of the Purdue Improved Crop Storage (PICS) bags as a post-harvest storage solution for biofortified maize. The specific focus of this research was to monitor retention of provitamin A and other carotenoids in two biofortified maize genotypes (OPVI and OPVII) as well as storage effect on flour functionality. Finally, a preliminary assessment of the impacts of storage on carotenoid bioaccessibility was completed to begin to translate findings to practice.

Maize grain from 2016 harvest was stored at ambient conditions for eight months in either PICS bags with or without an O_2 scavenger, (PICS-oxy) and (PICS-noxy), respectively and compared to storage in common polypropylene woven bags (control). After 4 months of storage carotenoid content was significantly higher ($p<0.05$) in PICS-oxy compared to PICS-noxy and woven bags demonstrating the importance of entrapped oxygen on maize carotenoid degradation. Furthermore, differences in carotenoid stability between maize genotypes were observed with OPVI having higher retention than OPVII. After 8 months, carotenoid retention remained dependent on storage bag and genotype with retention being greater in PICS-oxy and PICS-noxy compared to woven bags. However, final levels after 8 months were more similar between storage methods. Overall, oxygen content and genotype were found to be determining

factors in the effectiveness of PICS to mitigate carotenoid degradation during post-harvest storage of maize.

While reducing the rate of carotenoid degradation during postharvest storage of biofortified maize is important, success of biofortified maize is also dependent on consumer adoption of these grains and their performance in traditional food preparation. Assessment of the rheological and functional properties of these two biofortified maize genotypes as a function of post-harvest storage was completed to assess the impact of post-harvest storage in PICS bags on flour functionality and rheological properties for the two biofortified orange maize genotypes and a control white maize genotype. Flour pasting profiles were assessed initially and at 4 and 8 months. After 8 month storage in woven and PICS bag, OPVI and OPVII produced porridges with similar viscosities to their initial viscosities regardless of postharvest storage type. White maize viscosities progressively decreased with storage and were significantly lower ($p<0.05$) in woven compared to PICS storage. Sequestration of oxygen (PICS-oxy) had modest but significant effects ($p<0.05$) on key pasting parameters including peak and final viscosities. These results suggest that oxygen sequestration has a critical effect on final flour functionality. DTT treatment partially restored flour pasting profiles suggesting disulfide linkages may modify pasting profiles of flour. There was also an increase in free ferrulic and *p*-coumaric acids during storage which may have contributed to observed decreases in porridge viscosities. Evidence of this was found through Raman spectroscopy with spectral intensity at both $478cm^{-1}$ and $2911cm^{-1}$ decreasing with storage suggesting the potential for structural changes induced by storage on starch polymer. While storage in PICS bags does not seem to adversely affect flour functionality it may provide some additional economic benefit resulting from requiring proportionally less flour to achieve similar final viscosities as flour from woven bag stored grains.

Finally, the effect of postharvest storage on bioaccessibility of carotenoids was explored using experimental wet cooked porridges made from ‘fresh’ and stored grains using an established three stage in-*vitro* digestion model. Relative carotenoid bioaccessibility (% micellarization) was generally higher in less viscous porridge made from grains stored in woven bags compared to porridge from initial or PICS bags stored grains suggesting that higher viscosity might partly explain lower relative bioaccessibility in porridge from grains stored in PICS bags. Absolute carotenoid bioaccessibility from experimental porridge was dependent on

carotenoid species and storage system. Extrapolation of relative bioaccessibility (%) to absolute bioaccessibility (µg/g flour) suggests that fresh grains and their corresponding porridges would provide more absolute bioaccessible carotenoids compared to stored grains despite some improvement in relative accessibility. As such, storage losses remain the main factor impacting total available carotenoids and should continue to be an area of focus for future mitigation. With the potential to minimize post-harvest losses, improve carotenoid retention and provide a product with improved cooking performance, PICS bags do appear to offer a viable storage alternative to improve both food and nutrition security in developing countries.

CHAPTER 1. REVIEW OF THE LITERATURE

1.1 Introduction

tamin A deficiency (VAD) impacts one third of preschool children and 15% of pregnant women globally leading to an estimated 250,000 to 500,000 of children with VAD related childhood blindness (WHO, 2009; Tumuhimbise at al., 2013). Strategies to alleviate VAD rely on vitamin A supplementation and food fortification (where practical) despite having disadvantages of high cost, low rural coverage and compliance that make sustainability challenging (Bouis et al., 2013). Also, long term effectiveness remains in question. For example, despite having 80% of coverage globally with high dose vitamin A supplementation for the past 20 years, VAD is estimated to be decreasing only slowly. There are suggestions that this strategy is losing relevance due to changing disease pattern such as measles and diarrhea (Mason et al., 2015). Sustainable alternative matching local population norms are needed. Biofortification of staple crops is considered one such alternative strategy that presents an economical and potentially sustainable path relative to the primarily donor funded supplementation and fortification programs (Nestel et al., 2006). The potential of biofortification is believed to be high in regards to addressing key limitations of traditional programs as a result of their focus on leveraging staple foods that are consumed in large quantities by broad portion of the population (Nestel et al., 2006).

Maize is one such staple crop that provides food for over 900 million people located mainly in sub-Saharan Africa, Mexico and Central America (Pixley et al., 2013). Maize consumption patterns are variable from region to region but still remain a main source of nutrition globally. Maize consumption per person per day in Africa ranges from 52 gram in Uganda to 329 gram in Lesotho. Consumption is also higher in Region of Americas, South East Asia, European and Mediterranean regions (Ranum et al., 2014). While remaining major staple, common varieties of typical white and yellow maize remain low in some shortfall micronutrients (vitamin A, iron and zinc), they still remain the major source of carbohydrate and protein in the diets of developing countries. Yellow and orange maize are, however, a rich source of many health promoting bioactive compounds (phytochemicals) such as carotenoids including xanthophylls (lutein and zeaxanthin) and to a certain extent provitamin A carotenoids (β-carotene and β-cryoptoxanthin).

This natural genetic diversity of carotenoids in maize has made it a logical target for successful biofortification programs for provitamin A carotenoids compared to other cereals. Different genotypes have been developed with high level of provitamin A carotenoids at Purdue (9-16 μg/g dw) and in Zambia (12-15μg/g dw) (Ortiz et al., 2016; Mugode et al., 2014).

Although maize biofortification is a promising strategy for combatting VAD in developing countries (Bouis et al., 2013), it must still overcome specific challenges in order to fulfill the potential. This includes, addressing issues of post-harvest losses of provitamin A carotenoids and ultimate quality and performance of biofortified maize in consumer foods. It is a common practice in most countries to store maize for extended period post-harvest. However, over 80% of provitamin A carotenoids have been reported to be lost during postharvest storage of elite biofortified maize genotypes (Ortiz et al., 2016) reducing the potential effectiveness of biofortification programs. Strategies to manage such losses are considered as critical as further gains in provitamin A levels of biofortified in maize.

While there have been significant advances in adoption of biofortified orange maize in communities in Sub-Saharan Africa (Bouis and Saltzman, 2017), the potential for integration in the local food supply also remains dependent on product performance of biofortified relative to traditional maize cultivars in local communities. Nutritionally there is great potential as there is evidence that vitamin A equivalence of β-carotene derived from biofortified maize is potentially higher (6.48:1) than from conventional vegetable sources (12:1) as defined by Institute of Medicine (IOM) (Li et al., 2010). Overall, these observations support the potential for biofortified maize to directly impact the health of at risk populations with effective adoption. Realizing this potential still requires a better understanding of the ability of these emerging biofortified grains to perform in local processed and in home preparation and mitigation of postharvest losses in provitamin A levels or alteration of bioavailability/bioconversion.

Studies have shown that carotenoids from maize are generally considered to be highly bioaccessible (Thakkar and Failla, 2008; Kean et al., 2008). However, this may be dependent on the type and extent of food processing (Kean et al., 2008). The majority of these studies has also focused on “fresh” grain and therefore do not account for physico-chemical or physiological changes that occur during grain storage that can impact both carotenoid levels as well as digestive release/bioaccessibility. As it is known that post-harvest storage can induce changes

that make grains more organized (Zhou et al., 2003) resulting in hardening of cell walls (Swamy et al., 1978) and alteration of cooking quality, it is possible that such impacts to the food matrix could impact carotenoid bioaccessibility and by extension bioavailability. Such effects must be better understood in order to predict how postharvest storage and processing can be best leveraged to optimize the effectiveness of biofortified maize in specific populations.

While the nutritional value and potential impacts of biofortified orange maize relative to traditional white maize are documented, it is still important to consider that adoption of these improved grains is most dependent on consumer acceptability of such new grains. Information on product performance and quality attributes of many of the elite biofortified maize genotypes has been reported but generally is still lacking in detail. The lack of such information is critical to development of consumer based strategies and could, in fact, explain the varied perceptions regarding quality and acceptability of products from biofortified maize (Muzhingi at al., 2008; Nuss et al., 2012; Pillay et al., 2011). For example, several studies have documented similarities between macronutrient composition of biofortified and traditional white maize (Oluba and Oredokun-Lache, 2018; Li et al., 2007) including protein (Pillay et al., 2013). However, biofortified orange maize was reported to have significantly lower glycemic index than white maize (Oluba and Oredokun-Lache, 2018) suggesting that the presence of carotenoids, absent in white maize, or other differences not described could be impacting starch structure/functionality. These product attributes (e.g. viscosity) might also have impacted the starch digestibility and by extension, the glycemic index. In any case, the absence of clear comparisons of biofortified maize with more common white maize varieties for consumer centric product attributes such as cooking quality, remains a challenge for those interested in translation of these grains to traditional and even new product strategies that can be accepted by consumers. Furthermore, insights in effective post-harvest storage systems that can both preserve carotenoid levels and important product quality are needed.

Considering these needs, the efforts in this dissertation were designed to assess these two needs by evaluating the effectiveness of a cost effective post-harvest storage system, the Purdue Improved Crops Storage (PICS) bags, already deployed in developing countries, as a method to preserve carotenoid content and cooking quality of two elite biofortified maize genotypes. This literature review will cover: an overview of the cause of vitamin A deficiency, explain maize

carotenoids and their provitamin A activity, biofortification strategies for maize aimed at increasing provitamin A carotenoids. In addition the review will discuss storage of biofortified maize and retention of carotenoids post-harvest, effect of storage on physicochemical properties of cereal grains and how these effects could potentially affect carotenoid bioaccessibility from stored grains. Lastly, the review will discuss consumer acceptability of biofortified maize and potential challenges to translation of biofortified maize to at risk population

1.2 Vitamin A deficiency

Vitamin A (VA) as retinol is structurally an unsaturated C40 hydrocarbon structurally containing a β-ionone ring to which isoprenoid chain is attached (Figure 1.1). It is an essential micronutrient derived primarily from animal products (meat, eggs and dairy) or from provitamin A carotenoids including β-carotene from plant based sources (Fragoso et al., 2012). Collectively, retinol, retinal and retinoic acid are classified as retinoids, compounds which share structural similarities with vitamin A. Vitamin A is required for normal growth and development, reproduction, differentiation of cellular epithelium, body immunity and cell division (Edem et al., 2009). Inadequate intake of vitamin A leads to Vitamin A deficiency (VAD) which is characterized by ocular disorders such as night blindness, impaired cellular differentiation, reduced resistance to infection, anemia and depressed immune function and finally death (Fragoso et al., 2013). Symptoms may be reversed by acute vitamin A supplementation or alterations in the diet to include vitamin A or provitamin A rich foods. However, long-term deficiency can lead to effects that can be hard to reverse.

Globally, approximately one-third of preschool-age children and 15 % of pregnant women are estimated to be vitamin A deficient (WHO, 2009). The problem becomes more severe particularly in the developing countries whose poor populations rely on a single staple crop for their sustenance. In this case, VAD is the most common cause of childhood blindness with an estimated 250,000 to 500,000 children impacted each year with a mortality rate of 50% within 12 months of losing their sight (Tumuhimbise at al., 2013). The problem is attributed to a general lack of dietary vitamin A and reliance on plant based diets with staples that provide limited amounts of provitamin A carotenoids. Specifically, cereals and tubers such as maize, cassava, millet, sorghum and rice are normally vitamin A poor. In this context, biofortification is

a logical strategy to address this gap in nutrient density of staple cereal foods and, as such, complement supplementation and food fortification programs as a way to combat VAD in developing countries by increasing dietary exposure to provitamin A carotenoids.

1.3 Carotenoids and provitamin A activity

Carotenoids are naturally occurring pigments found in most fruits and vegetables, plants, algae, and photosynthetic bacteria. In humans, carotenoids have several health benefits including antioxidant activity, promotion of eye and brain development and health and perhaps most critical, as a precursor for vitamin A (provitamin A activity) (Liu, 2007; Eggersdorfer and Wyss, 2018). Carotenoids are well known to act as antioxidants in chemical and biological systems by virtue of the highly conjugated double bond structure (Figure 1) allowing for quenching of reactive oxygen species (Liu, 2007). However, physiologically they may be more important for their ability to stimulate endogenous antioxidant systems in the human body (Fiedor and Burda, 2014). Human beings cannot synthesize carotenoids and therefore carotenoids must be obtained from the diet and primarily from fruits, vegetables and in some cases biofortified cereals.

While over 600 carotenoid species exist in nature (Stahl and Sies, 2003), most common carotenoids in diet and by extension, observed physiologically in human fluids and tissues include lycopene, α-carotene, β-carotene, β-cryptoxanthin, lutein and zeaxanthin (Eggersdorfer and Wyss, 2018). Of these carotenoids, α-carotene, β-carotene, and β-cryptoxanthin possess provitamin A activity by virtue of the fact that they can be metabolized to retinol through the action of β-carotene 15′15 dioxygenase 1 (BCO1) in the intestine and liver of humans (Lietz et al., 2012; Luo and Wang, 2012). This is by virtue of their structure having at least one un-substituted β-ionone ring at either one or both end of the 11-carbon polyene chain (Figure 1.1). Eccentric cleavage of carotenoids has also been observed to be carried by β-carotene 9′10′ dioxygenase 2 (BCO2) (Lietz et al., 2012). Similarly, BCO1 cleave α-carotene and β-cryptoxanthin and produce one molecule of retinol and therefore both α-carotene and β-cryptoxanthin have half the vitamin A activity of β-carotene. In addition to provitamin A carotenoids, the xanthophylls lutein and zeaxanthin which are abundant in maize, are the critical dietary carotenoids that have established functions as macular pigments accumulating in the

macular region (yellow spot) of the human retina. These pigments play a role both in eye and neuronal development and protect against age-related macular degeneration (Johnson, 2014).

Food based strategies to combat VAD have included diversification of diets of at risk groups such as pregnant women and children with bright orange or dark green fruits and vegetables that are rich sources of provitamin A carotenoids (Seo et al., 2005). The quantity of provitamin A carotenoids needed to alleviate VAD through biofortification depends both on their bioavailability and bioconversion, which is influenced by a number of factors including food matrix, processing, type and amount of fat and nutritional and pathophysiological status of the individual (Van het Hof et al., 2000; Goltz et al., 2012; Titcomb et al., 2018).

Effort has been made to alleviate VAD through strategies that seek to increase vitamin A intake (Seo et al., 2005). These strategies include vitamin A supplementation for under-five children, food fortification with vitamin A and promotion of dietary diversification that include vitamin A rich foods. While success with these strategies have been reported (Bouis et al., 2013), problems with targeting and reaching out to at risk population and low compliance have been serious setbacks (Nestel et al., 2006). To overcome these challenges strategies such as biofortification of staple food such as maize were initiated in order to enhance nutrient content of these staple foods as cost effective and sustainable solution.

1.4 Maize carotenoids

Maize is a logical crop for biofortification with carotenoids as it exhibits considerable natural variation in carotenoid content and profiles. Some genotypes accumulate as high as 66.0 μg/g of total carotenoids while others have almost none (Harjes et al., 2008). Yellow maize kernel carotenoids are present in different isoforms, including two carotenes, α- and β-carotene, and four xanthophylls, β-cryptoxanthin, zeinothanthin, zeaxanthin, and lutein (Watson, 1962; Weber, 1987). The predominant carotenoids in maize kernels are typically lutein and zeaxanthin, followed by β-carotene, β-cryptoxanthin, and α-carotene (Figure 1.1). Generally, provitamin A carotenoids constitute only 10–20 % of total carotenoids in maize, whereas zeaxanthin and lutein each commonly represent 30–50 %. The amounts of provitamin A in traditional yellow maize varieties range widely from 0.25 μg to 2.5 μg g^{-1} dry weight. Most yellow maize contains less than 2 μg/g while white maize has almost no provitamin A carotenoids (Pixley et al., 2013). In

typical maize, concentrations of provitamin A carotenoids, range from 0 to 1.3, 0.13 to 2.7, and 0.13 to 1.9 nmol/g, respectively (Kurilich and Juvik, 1999). Although β-carotene has the highest provitamin A activity, it is present in a relatively low concentration (0.5–1.5 μg/g) in most yellow maize grown and consumed throughout the world (Harjes et al., 2008).

Different biofortified maize genotypes have been developed/released by different institutions (Table 1.1). These genotypes vary in total carotenoid as well as total provitamin A contents. Genotypes released by Purdue University seem to have higher total carotenoids compared to those released in Zambia. However, all biofortified orange maize genotypes have significantly higher carotenoid contents than white genotypes presented in table 1.1.

Table 1.1. Total and provitamin A carotenoid contents of some select white and biofortified maize genotypes

Genotype	Releasing Institution	Total carotenoid (μg/g dw)	Provitamin A carotenoids (μg/g dw)	Reference
White (Landrace)	Malawi	2.12	0.28	Hwang et al., 2016
White(TZL COMP4 C2)	Nigeria	1.52	0	Oluba and Oredokun-Lache, 2018
2012 Orange ISO Selected A	Purdue University/Indiana	61.1	8.5	Ortiz et al., 2016
(CI7× DE3)× 2010-Orange-Isolation	Purdue University/Indiana	47.3	12.3	Ortiz et al., 2016
CI7 ×DE3	Purdue University/Indiana	39.8	16.1	Ortiz et al., 2016
Hi27× CML328	Purdue University/Indiana	40.1	9.2	Ortiz et al., 2016
GV664A	Zambia	20.3	7.6	Taleon et al., 2017
10 MAK 7-5	Makhathini Research Station/South Africa	23.0	7.3	Pillay et al., 2014
10 MAK 7-7	Makhathini Research Station/South Africa	26.4	7.7	Pillay et al., 2014

Table 1.1 continued

Genotype	Releasing Institution	Total carotenoid (µg/g dw)	Provitamin A carotenoids (µg/g dw)	Reference
10 MAK 7-8	Makhathini Research Station/South Africa	22.3	8.3	Pillay et al., 2014
BRY 9928 DMR SR-yellow	IITA/Nigeria	12.7	3.7	Oluba and Oredokun-Lache, 2018
MW5021-Mthikinya (Landrace)	Malawi	44.3	12.3	Hwang et al., 2016
GV662A (HP1002)	ZARI/Zambia	30.8	12.9	Mugode et al., 2014
GV665A (HP1005)	ZARI/Zambia	24.5	14.9	Mugode et al., 2014
HP1001	ZARI/Zambia	24.4	12.8	Mugode et al., 2014
HP1003	ZARI/Zambia	25.5	12.2	Mugode et al., 2014
2016 Orange Iso (OPVI)	Purdue University/Indiana	54.3	8.9	Nkhata et al., 2019
2016 Mosley (OPVII)	Purdue University/Indiana	45.4	9.7	Nkhata et al., 2019

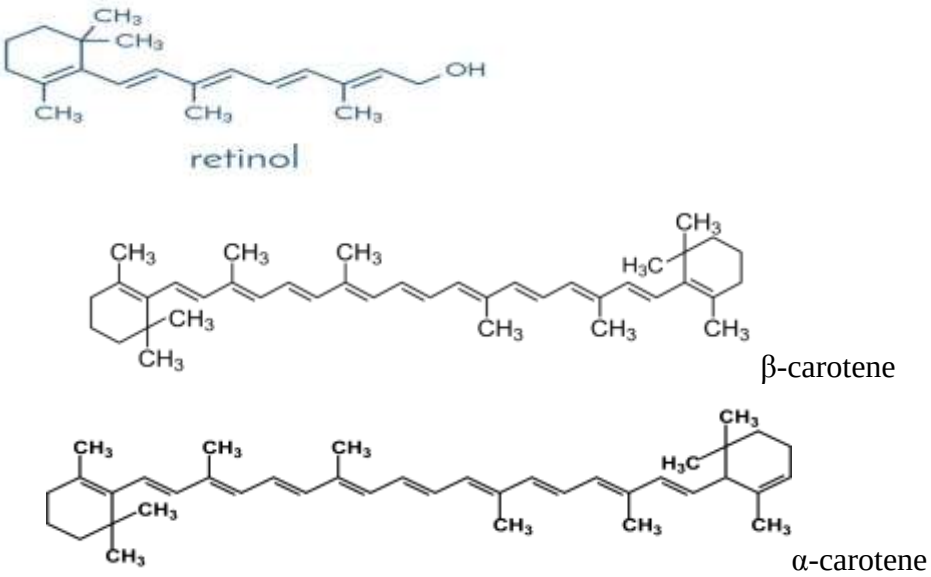
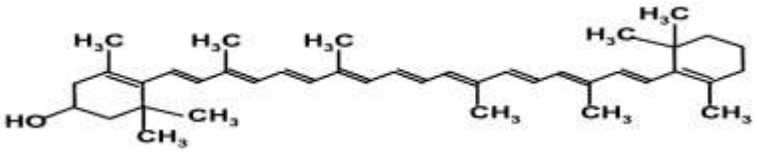
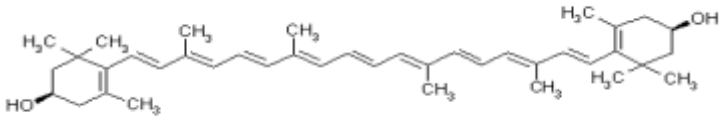


Figure 1.1. Structures of retinol and major carotenoids present in maize grain

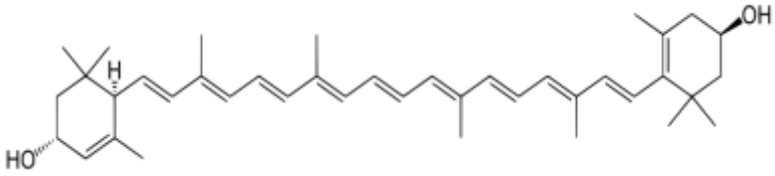
Figure 1.1 continued



β-cryptoxanthin



Zeaxanthin



Lutein

1.5 Biofortification strategies for maize for provitamin A carotenoids

Biofortification is a process of breeding staple food crops for higher nutrient content/accumulation (Bouis et al., 2013). Generally, biofortification of staple crops has been accomplished by two ways; conventional and marker assisted breeding or through transgenic modification. Conventional breeding practices include artificial mating or cross pollination of two or more plants that have desirable traits. Transgenic approaches can involve either natural transferring of foreign genetic material that encodes desirable traits, in this case higher provitamin A synthesis or accumulation. A number of genetic engineering techniques can be leveraged to achieve this goal including gene editing such as CRISPR (clustered regularly interspaced short palindromic repeats)/CRISPR-associated (Cas) systems (Gaj et al., 2013). New varieties from these techniques may not be subjected to the same challenges as "traditional" transgenic varieties because they are not fully considered genetically modified crops (in the US) and therefore may have potential to revolutionize the way these biofortified crops are perceived (Gaj et al., 2013; Hefferon, 2015).

Both conventional and transgenic practices have been used to increase carotenoid content in maize (Aluru et al., 2008; Nestel et al., 2006). However, conventional breeding remains the primary strategy used in many biofortified crops because it allows for more rapid adoption of

materials globally without as many regulatory hurdles. As such, HarvestPlus, a leading institution in staple crop biofortification has allocated 85% of its budget to conventional bleeding and has focused on combining high micronutrient density traits with high yield (Nestel et al., 2006). While promising it is challenged by limited variability in the plant genome and the longer lead/development times required to generate cultivars with desired traits (Hefferon, 2015).

In contrast to conventional approaches, transgenic strategies provide shortened timelines for development. These strategies have commonly targeted the overexpression of the bacterial genes for phytoene synthase and the enzymes (phytoene desaturase and *z*-carotene desaturase) that catalyze the four desaturation steps in the carotenoid biosynthesis pathway resulted in an increase of total carotenoids up to 34-fold with preferential accumulation of β-carotene in the maize endosperm (Aluru et al., 2008). Unlike conventional breeding which is limited to closely related species and takes a long time to achieve targeted results, transgenic breeding allows leveraging of useful genes from a wide range of living sources and are reproducible from many generations (ISAAA.org). Moreover, characteristics of interest do not always exist in related species.

The high β-carotene trait was found to be reproducible over at least four generations. Analyses indicated that up regulation of the endogenous lycopene β-cyclase was responsible for the accumulation of β-carotene (Rodriquez-Amaya et al., 2011). Studies have shown that it is possible to combine both high micronutrient density traits with high yield in all food crops that have been studied (Graham et al., 1999).

1.6 Storage of biofortified maize and retention of carotenoids post-harvest

While the target for maize biofortification has been focused on increasing provitamin A carotenoid, with specific focus on β-carotene (Titcomb et al., 2018), consideration has also been given to other factors that represent hurdles to this process. For example, consideration of genetic differences in the post-harvest stability of carotenoids has been reported (Ortiz et al., 2016) suggesting the potential for new targets that can be breed for to enhance nutrient retention. Also, carotenoid profiles can be designed to leverage enhanced bioavailability. For examples, β-cyptoxanthin is typically found in lower contents compared to β-carotene in most maize genotypes, however, it has been suggested that this carotenoid species is more bioavailable than

β-carotene (Burri et al., 2011; Burri et al., 2016). Strategies to develop maize genotypes with higher β-cryptoxanthin have shown promise in biofortification (Titcomb et al., 2018).

However, variability in post-harvest storage and carotenoid retention are critical to establishing viable biofortified lines as losses postharvest represent a major hurdle in implementation of these grains. Currently storage of biofortified maize is done in a fashion similar to white maize because presently there is no alternative or economical way of storing these improved grains in developing countries that have shown any promise in controlling carotenoid degradation post-harvest. The main storage techniques currently used (Figures 1.2) are primarily intended to protect grains from storage pests and mold infestations and have demonstrated a limited capacity to maintaining the nutrition quality of stored grains especially those related to carotenoids. These types of storage are not completely aligned with preserving provitamin A carotenoid content and significant losses of carotenoids in these storage methods (or similar systems) have been reported (Mugode et al., 2014; Taleon et al., 2017). In fact, losses of up to 90% of carotenoids during 12 months post-harvest storage under controlled conditions have been reported (Ortiz et al., 2016) that mirror many of the expected losses in traditional storage systems.

Different storage structures and systems leveraging altered or controlled environmental conditions have been tested with no or limited benefits compared to ordinary storage in sacs or bags (Mugode et al., 2014; Burt et al., 2010; Taleon et al., 2017). In most developing countries post-harvest losses of grains to insect or mold damage remain a serious problem, with nutritional losses representing a distant concern. In this regards, the Purdue Improved Crop Storage (PICS) bags were developed to address insect and mold spoilage of staple crops including grains and introduced in developing countries to stem this problem (Baoua et al., 2014). PICS bags are designed with two high density polyethylene (HDPE) liners (80 μm) inserted inside an outer woven polypropylene sack. PICS functions by virtue of a bio-generated atmosphere based on reduction of oxygen and increase in carbon-dioxide inside the bag. When oxygen becomes sufficiently low insects die of desiccation (Njoroge et al., 2014). This mechanism may hold potential benefits to enhancing carotenoid stability in stored grains as it has been established that at low level of oxygen it has been observed that the rate of carotenoid degradation can be

reduced if oxygen is controlled, as it is a major factor in carotenoid oxidative degradation during storage in agriculture (Bechoff et al., 2010).

Carotenoids being highly unsaturated compounds prone to oxidation tend to degrade forming products such as epoxides, apocarotenals and apocarotenones as well as *cis*-isomers. These degradation products lead to a loss of provitamin A activity and potentially altered bioavailability (Anan et al., 2005). These reactions are dependent on availability of oxygen and are enhanced by light, heat, presence of metals and various enzymes such as peroxidases and deoxygenases (Pixley et al., 2013). Carotenoids are highly susceptible to degradation during storage. Mugode et al (2014) reported up to 60% degradation of carotenoids during storage for six months in woven bags under ambient conditions (16-27 ^{0}C). During storage of maize provitamin A losses can generally vary from 7 to 45% depending on variety with fastest degradation occurring during the first 2 months (Pixley et al., 2013). Degradation is highest in milled flour stored in translucent permeable packaging than for whole kernel or finely milled flour stored in vacuum sealed bags (Taleon et al., 2017). The increased degradation rate in milled flour is due to the destruction of cellular structure during milling which increases surface area and porosity and thus leads to increased exposure of the carotenoids to oxygen and pro-oxidant environment (Rodriquez-Amaya et al., 2011).

Interestingly, decreases in oxygen and increase in carbon dioxide content in PICS systems are achieved with the help of activities of insect pests placed inside the bags (Njoroge et al., 2014). In fact storage of biofortified maize in PICS bags has been reported to provide some improvement of carotenoid stability over 6 months relative to common polypropylene woven bags (Taleon et al., 2017). In this preliminary study, PICS bags without scavengers achieved ~6% more provitamin A carotenoid retention (57%) compared to woven bags (51%) after 6 months storage of biofortified maize (Taleon et al., 2017). Storage of biofortified maize in metallic silos with a burning candle achieved similar levels of provitamin A carotenoid retention (57%) to PICS bags while metallic silos without candle achieved similar provitamin A carotenoid retention (50%) to woven bags (Taleon et al., 2017). However, metallic silos are expensive compared to PICS bags and farmers may not afford them. These data are a promising sign that the economic PICS system could provide oxygen sequestration and prove to be a method to maintain nutritional quality for biofortified maize.

Figure 1.2.Traditional methods of storing maize grains in developing countries. A. Traditional metallic silo. Source: http://edepot.wur.nl/446120 . B. Traditional granary (nkhokwe). Source: www.cimmyt.org . C. Woven bags. D. PICS bags.

1.7 Effect of food processing on stability of carotenoids

In addition to post harvest storage, food processing and in home preparations have been documented to impact carotenoid retention in select foods including biofortified maize. Processing of maize includes milling and subsequent transformation into food products such as wet cooked porridges, breads, snacks etc. These processes rely on physical and chemical unit operations including mechanical matrix disruption and heat treatment that directly affect carotenoid stability (Ortiz et al., 2018). For examples, decortication resulted in 10% carotenoid loss while dry milling resulted in 28% loss of provitamin A carotenoids after 90 days of storage.

Extrusion processing at 35% moisture content resulted in 70-93% retention of provatimain A caroytenoids (Ortiz et al., 2018). Fermentation of biofortified maize genotypes for 24 and 72 hours retained 60-100% of provitamin A carotenoids while further fermentation time (120 hours) significantly decreased carotenoid retention to between 27-48% depending on genotypes (Ortiz et al 2017). Approximately 55% of carotenoids were retained after oven drying of fresh maize (Burt et al., 2010) while storage for 6 months at room temperature retained 58% (Weber et al., 1987). Dark storage at 35% relative humidity for 4 and 18 months retained between 60-65% and 39-78% of carotenoids, respectively, (Burt et al., 2010). Dough making for 53 min, chip frying at 175 ^{0}C for 1 minutes and tortilla baking at 280 ^{0}C for 1 min resulted into 57%, 18% and 32% retention of β-carotene, respectively, (De La Parra et al., 2007). Nixtamalization and frying of Mexican maize food products retained 64% of total carotenoid (Lozano-Alejo et al., 2007). Heat treatment during porridge making significantly decreased carotenoid retention compared to heat treatment during baking of bread and extrusion of maize puff (Kean et al., 2008). Interesting, as processing is known to disrupt the food matrix, making carotenoids more accessible to chemical degradation, they also can enhance the bioaccessibility (Ryan et al., 2008) and improve carotenoid bioavailability (Rodriquez-Amaya et al., 2011). Processing softens or breaks membranes and cell walls and denatures proteins complexed with carotenoids, facilitating the release of these compounds from the food matrix during digestion. Therefore, processing conditions should be optimized to increase bioavailability without provoking significant degradation of the carotenoids.

1.8 Effect of storage on physicochemical properties of cereal grains

As described above, storage of maize is a common practice in many households in developing countries. Maize grains are stored in sacs, bags, mud and metallic silos and traditional granaries called *nkhokwe* (Figure 1.2). It is also a common practice to store maize for many months. During storage, grains undergo several physicochemical changes through exposure to temperature, light and oxygen that affect cooking properties of derived flour (Swamy et al., 1978). Some of these changes are known to result from progressive decrease in apparent amylose content, hardening of cell walls, development of free fatty acids and subsequent complexing with starch and proteins (Swamy et al., 1978). Extensive literature about these changes exists in rice (Keawpeng and Venkatachalam, 2015; Patindol et al., 2005; Hamaker and

Griffin, 1990; Dhaliwal et al., 1991; Awazuhara et al., 2000; Setiawan et al., 2010; Swamy et al., 1978) with a more limited amount of information available on maize. These changes are not believed to be related to any structural changes of starch but rather due to structural changes of non-starch component surrounding starch granule within the food matrix (Teo et al., 2000, Kumar and Ali, 1991, Zhou et al., 2003). To confirm this, starch extracted from stored grains did not show ageing related changes shown by derived flour (Zhou et al., 2003; Kumar and Ali, 1991) indicating that these changes are not related to starch *per se*. However, starch related changes such as shift in amylose:amylopectin ratio and increased starch crystallinity have been reported in starch derived from stored grains (Awazuhara et al., 2000; Setiawan et al., 2010).

The changes over storage do impact flour functionality and pasting profiles. A decrease in peak viscosity (PV) over storage of rice was attributed to increased polymerization of protein during storage (Hamaker and Griffin, 1990; Patindol et al., 2005) which is believed to reduce starch granule ability to swell as the kernel became more organized (Zhou et al., 2003). When starch granule fully swells it ruptures and leaches amylose which results in increases in PV (Patindol et al., 2005). Interestingly, increases in PV from storage of wheat whole meal was attributed to free fatty acid release during storage and presence of potential complexes with amylose (Copeland et al., 2009; Salman and Copeland et al., 2007). In milled rice it was attributed to changes in diastase enzymes and free fatty acid released (Dhaliwal et al., 1991). There was a progressive increase in free fatty acid during storage which increased final viscosity (FV) due to stronger free fatty acid-amylose re-association (Keawpeng and Venkatachalam, 2015; Copeland et al., 2009; Salman and Copeland et al., 2007). FV is a function of starch retrogradation occurring when amylose molecules re-associate with each other through hydrogen bonding to form a double helix (Alcazar-Alay and Meireles, 2015). Retrogradation indicates the ability of starch to form a viscous gel (Sandhu and Singh, 2007) and is an important parameter as it determines the serving viscosity of the paste (ie porridge or *nshima*) and has significant influence on perceived texture and mouth feel of the porridge.

Different enzymatic activities also play a role in changes in viscosity from grain storage. Previous studies have attributed increase in PV to progressive decline in α-amylase activity of stored rice (Dhaliwal et al., 1991). However, in stored wheat grains α-amylase activity was negligible despite the increase in PV after 84 days of storage (Fierens et al., 2015) suggesting

that α-amylase activity alone cannot explain all changes in PV. Other enzymes such as lipoxygenase, lipases and proteases which remain active during storage are believed to play a role by altering the oxidative environment (Dhaliwal et al., 1991; Fierens et al., 2015). These changes are likely going to affect flour functionality and textural properties of derived products depending on storage system. It is therefore hypothesized that storage of biofortified maize will result in storage changes that may impact product quality and acceptability.

1.9 Bioaccessibility and bioavailability of carotenoids from different food matrices

Bioaccessibility is a termed used to define the proportion of carotenoids from a food that are released during normal digestion and transferred to bile salt mixed micelles and made available for absorption (Goltz et al., 2012). This is a precursor and predictor of actual bioavailability which is defined as the proportion of carotenoids absorbed from a specific food and is made available in the body for utilization, metabolism and/or storage (Parker et al., 1999; Goltz et al., 2012). Generally, relatively polar xanthophylls such as lutein, zeaxanthin and β-cryptoxanthin have been reported to have higher bioaccessibility and bioavailability than apolar carotenes such as β-carotene, α-carotene and lycopene (van het Hof et al., 1999; Castenmiller et al., 1999). The higher bioaccessibility of xanthophylls emanates from their location on the outer surface of micelles where they can be easily accessed by hydrolytic enzymes resulting in greater absorption (La Frano et al., 2014). Moreover, the release of xanthophylls into an aqueous environment is higher than that of β-carotene due to lower lipophilicity (van het Hof et al., 1999a). Unlike carotenes that are found embedded within the micelle, xanthophylls are found in the outer layer of micelles where they may be easily accessed by digestive enzymes. Carotenoids bioavailability ranges widely across food matrices. For example availability from maize (16.7%), orange fleshed sweet potato (0.6-73%), carrots (19-34%) and broccoli (22-24%) are reportedly higher than those from green leafy vegetables (3-6%), (Thakkar and Failla, 2008; Bechoff et al., 2011; Failla et al., 2009; Mills et al., 2009; Brown et al., 1989; Micozzi et al., 1992; De Pee et al., 1995; Torronen et al., 1996; Castenmiller et al., 1999; van het Hof et al., 1999).

Bioaccessibility/bioavailability of maize carotenoids can vary depending on type of processed maize product consumed. For example, micellarization efficiency of xanthophylls from yellow cornmeal extrude puff was higher (63-69%) than from yellow cornmeal porridge

(48%) (Kean et al., 2008). In the same study micellarization efficiency of xanthophylls from whole yellow cornmeal was highest in bread (85%) and lower in extruded puff (46%) and porridge (47%). β-carotene had the lowest micellerization efficiency in puff and bread (11-23%) with higher in porridge (40-63%) (Kean et al., 2008).

Co-consumption of carotenoid rich food with fats increases carotenoid bioavailability (Goltz et al., 2012). Presence of fats initiates excretion of bile salts which is very important for formation of micelles. Studies have shown that both amount and type of fats affect carotenoid absorption with optimal absorption previously believed to occur when 3-6 g (Jalal et al., 1998; Van het Hof et al., 2000) of fats are included in the diet. Recently, significantly high level of 20g fat was found to be more efficient in increasing carotenoid bioavailability than 3 and 8g when subject consumed test salad (Goltz et al., 2012). Fats sources rich in monounsaturated fatty acids such as canola oil increased carotenoid bioavailability more than saturated fatty acid rich butter (Goltz et al., 2012). Though both amount and type of fat affect carotenoid bioavailability, amount exert the greatest effect. Unfortunately, diets for most developing countries do not contain adequate fat (Micha et al., 2014) a factor which may reduce carotenoid bioavailability from most single meals.

Bioavailability of β-carotene from biofortified maize has been found to be high as 6.48 μg was found to provide vitamin A activity equivalent to 1 μg retinol in a study involving 6 healthy non-smoking women (Li et al., 2010). Institute of Medicine (IOM) established an retinol activity equivalence (RAE) value of 12:1 for β-carotene and 24;1 for α-carotene and β-cryptoxanthin from food derived primarily from mixed vegetable sources in the US diet (Food and Nutrition Board, Institute of Medicine, 2001). The 6.48:1 bioconversion factor for biofortified maize is higher than that established by IOM. Based on several factors including postharvest losses, bioavailability and bioconversion, a target of 15 μg provitamin A per gram dry weight of kernel was established for biofortification of maize (Taleon et al., 2017)). This level is considered sufficient to address up to 50% of the EAR of vitamin A in populations that consume a large quantity of maize (Ranum et al., 2014) and highest VAD (WHO, 2009).

Storage of maize alters different physicochemical properties of the grains that affect flour functionality. These changes also reduces protein and starch digestibility (Rehman et al., 2002). It is not well understood how ageing-related changes may affect carotenoid bioavailability. Low

starch and protein digestibility may reduce carotenoid extractability and ultimate bioaccessibility since over 80% of carotenoids are found in starch-rich endosperm (Kean et al., 2008; Weber, 1987). Therefore, studies are required to understood how these ageing-related or storage changes might affect carotenoid bioaccessibility and ultimate bioavailability.

1.10 Consumer acceptability of biofortified maize

Biofortified maize is in two categories, yellow maize genotypes and recently introduced orange maize genotypes. While yellow maize has high carotenoids content including that of β-carotene than white maize, orange maize has higher carotenoid content than yellow maize and is therefore promoted for cultivation and consumption to alleviate VAD in developing countries. Yellow maize had existed as landrace or hybrid genotypes and had been mainly grown and utilized as animal feeds (Tumuhimbise at al., 2013). Occasionally, consumer acceptability tests are using 'provitamin A biofortified maize' to mean either orange, yellow or both genotypes (Pillay et al., 2011; Govender et al., 2014; Awobusuyi et al., 2016; Meenakshi et al., 2012). However, few studies had compared consumer acceptability of yellow vs white maize and orange versus white or yellow maize (Pillay et al., 2011; Stevens and Winter-Nelson 2007; Meenakshi et al., 2010; Nuss et al., 2012; Govender et al., 2014). While the more noticeable difference between white and orange maize is color the presence of carotenoids in biofortified crops seem to not only affect color but also texture, odor and taste (Tomlins et al., 2012; Talsma et al., 2017). Moreover, color did not affect acceptance of orange maize varieties in Mozambique (Schmaelzle et al., 2014; Alamu et al., 2015) while taste, texture and aroma did (Stevens and Winter-Nelson, 2007). The carotenoid effect on sensory properties is supported by recent study that showed proximate composition between white and biofortified maize genotypes were similar except for carotenoid content (Oluba and Oredokun-Lache, 2018).

White maize is generally preferred over yellow maize by broad categories of African population (Pillay et al., 2011; Stevens and Winter-Nelson, 2007). However, studies conducted in Zambia concluded that orange maize is likely to be accepted better by rural consumers (Nuss et al., 2012). Moreover, nutritional education campaigns translated into improved acceptance and willingness to pay for orange maize and that nutrition education was the single most important factor in determining household decision to purchase nutritionally enhanced orange maize

(Muzhingi et al., 2008). The study found that people will accept biofortified orange maize as long as they are made aware of the availability, economic and health benefits of the maize.

Majority of consumers preferred white maize over biofortified maize in large part due to a lack of knowledge on nutritional quality of orange maize (Muzhingi at al., 2008). It seems the preference of white over orange can be misguided because biofortified orange maize is mistaken for yellow maize which is mainly associated with food aid and animal feeds (Tumuhimbise at al., 2013) but also is generally considered to have poor sensory characteristics (Muzhingi at al., 2008). Though this was the case, one study indicated that negative perception of yellow maize does not affect the liking of orange maize (Meenakshi et al., 2010). Different studies have documented comparative acceptability of biofortified orange maize and white maize and data look promising (Table 1.2). Knowledge about the availability of vitamin A in yellow maize drove preference for the maize by study participants in Limpopo Province in South Africa (Khumalo et al., 2011). Biofortified orange maize was well accepted by children, caregiver and trial staff in a controlled randomized clinical trial in Zambia (Schmaelzle et al., 2014).

Table 1.2. Comparative consumer preference for products made from white and biofortified maize

Product	Country	Comparison	Preference	Subjects	Reference
Porridge	Zambia	white vs orange	similar	children	Nuss et al., 2012)
Porridge	KwaZulu-Natal. South African	white vs orange	similar	mothers (female caregivers)	Govender et al., 2014
Porridge	South Africa	white vs yellow	similar	primary school children	Pillay et al., 2011
Porridge	South Africa	white vs yellow	yellow	preschool chilren	Pillay et al., 2011
Porridge	South Africa	white vs yellow	white	secondary school children and adults	Pillay et al., 2011
Willingness to pay	Ghana	white vs orange	white	rural consumers	De Groote et al., 2010
Mahewu	South Africa	white vs orange	orange	rural farmers (adults)	Awobusuy et al., 2016
Fresh boiled maize	Nigeria	orange	scored 6.9/9 on hedonic scale		Alamu et al., 2015

Acceptability of biofortified orange maize could also be driven by farmers' economic gain. In Maputo, Mozambique, households that grew and therefore were predominantly consuming biofortified yellow maize were associated with lower level of household income (Tschirley and Santos, 1995). This could be related to the fact that consumers are willing to buy biofortified maize (orange or yellow) at lower price than white maize (Tschirley and Santos, 1995; Rubey and Lupi, 1997; De Groote and Kimenju, 2008; De Groote and Kimenju, 2011). Low prices might discourage farmers from growing biofortified maize. However, some participants accepted to trade off white maize for biofortified maize (Stevens and Winter-Nelson 2007). The major determinants for acceptance of biofortified maize in this study were household size, presence of children, dietary diversity and taste (Stevens and Winter-Nelson 2007). Nutrition education increased the price people were willing to pay for biofortified orange maize (De Groote et al., 2010; Meenakshi et al., 2012). In general, the literature to date indicates that biofortified maize would be accepted much more if the price is lower than that of white maize. On the contrary, one study conducted in Western and Eastern Kenya showed that willingness to pay for biofortified maize was 25% higher than white maize (De Groote and Kimenju, 2011).This acceptability is, however, variable depending on demography of the study population.

1.11 Challenges to translation of biofortified maize

Biofortification seems to be cost effective compared to food fortification and nutrient supplementation in reaching out the remotest population, therefore, addressing factors that may hinder its progress is of paramount importance. These factors include carotenoid stability during storage, product physical and sensory qualities and ultimate carotenoid bioavailability. Addressing loss of carotenoid during storage is critical and could be done through breeding of varieties that provide more stability to carotenoids. Devising a storage procedure that would reduce loss of carotenoids during storage is also critical to the success of biofortification as it would ensure higher contents of carotenoids are available for final consumers. Studies determining consumers preference for biofortified maize have been done albeit with mixed results and conclusion (Muzhingi at al., 2008; Tomlins et al., 2012; Talsma et al., 2017; Stevens and Winter-Nelson, 2007; Pillay et al., 2011; Meenakshi et al., 2010; Nuss et al., 2012; Govender et al., 2013; Schmaelzle et al., 2014). As the major difference between traditional white maize and biofortified maize is color, you would expect this to drive consumer preference. However

some studies (Schmaelzle et al., 2014; Alamu et al., 2015) have shown that color does not affect acceptance of orange maize varieties while texture does (Stevens and Winter-Nelson, 2007). This therefore provides a platform to characterize textural properties of biofortified maize that would drive consumer preference relative to ordinary white maize.

As already stated, storage induces physicochemical changes in grains that may have impact on carotenoid bioaccessibility. Moreover, biofortified grains are targeting developing countries where dietary fat consumption is very low (Micha et al., 2014; http://chartsbin.com/view/1156). Studies have shown that carotenoid bioavailability from vegetables was higher when co-consumed with fats (Goltz et al., 2012). Both type and amount of fats were important in increasing carotenoid bioavailability (Jalal et al., 1998; Van het Hof et al., 1999; Goltz et al., 2012) with significantly higher level of fats (20g) found to be more efficient in increasing carotenoid bioavailability than 3 and 8 g fats (Goltz et al., 2012). Unfortunately, achieving this level of fat in diets of most targeted developing countries could be a challenge. Co-consumption of biofortified maize and its derived products with fat-rich foods such as ground nuts, might be the most practical way of increasing fat intake and potentially increase carotenoid bioavailability form biofortified maize.

1.12 Experimental Aims

Considering the potential of high provitamin A biofortified maize to alleviate VAD in at risk populations, it is important to address key translational challenges including storage and food processing effects. While most studies are focusing on understanding stability of carotenoids in maize during post-harvest storage, few have studied economical ways that can be disseminated along with biofortified maize as potential solutions. It remains a critical challenge that requires careful consideration of both effective and deployable technologies that are suitable for storage of biofortified maize at minimal cost for preservation of carotenoids. In addition, carotenoid rich maize has to be accepted by a target population. This requires in depth consumer insights. However, prior to detailed consumer studies, comparative assessment of key attributes such as cooking performance of key biofortified maize genotypes would provide useful benchmarks for formulation of products or development of recommendations with which biofortified genotypes can be used to best emulate traditional maize function that is widely

accepted by consumers. This understanding of comparative performance attributes of biofortified maize flour relative to traditional white maize flour in food systems is critical. The inevitability of storing biofortified maize for later use makes investigation of the effect of ageing related changes on ultimate carotenoid bioavailability important to estimating potential for broader nutritional impacts.

In order to address all these specific gaps, the *overall objective* of this project was to characterize post-storage changes that occur to biofortified maize that may impact its nutritional and physical quality. To achieve the overall objective the following specific objectives were proposed;

Specific objective 1. **To assess the effect of oxygen sequestration on effectiveness of Purdue Improved Crop Storage (PICS) bags in reducing carotenoid degradation during post-harvest storage of two biofortified orange maize genotypes**. *Working hypothesis*; maize stored in PICS bags would have higher retention of carotenoids that those stored in woven bags and that by reducing oxygen levels inside the PICS bags, carotenoid retention would be further improved during postharvest storage.

Specific objective 2. **To determine the effect of Purdue Improved Crop Storage (PICS) bags on flour rheological properties and functionality**. *Working hypothesis*; Storage of maize in PICS bags would reduce ageing-related changes and subsequently alter pasting properties relative to storage in woven bags and this effect would be further enhanced by sequestering oxygen inside PICS bags.

Specific objective 3. **To compare bioaccessibility of carotenoids from stored (aged) and initial (none aged) biofortified maize.** *Working hypothesis*; ageing-related changes happening during storage of biofortified maize grains would reduce carotenoid bioaccessibility relative to initial grains.

CHAPTER 2. ASSESSMENT OF OXYGEN SEQUESTRATION ON EFFECTIVENESS OF PURDUE IMPROVED CROP STORAGE (PICS) BAGS IN REDUCING CAROTENOID DEGRADATION DURING POST-HARVEST STORAGE OF TWO BIOFORTIFIED ORANGE MAIZE GENOTYPES

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2.1 Abstract

Increasing adoption of biofortified orange maize in developing countries requires economical storage methods to manage product quality and carotenoid retention. This study assessed the utility of the Purdue Improved Crop Storage (PICS) bags with a specific focus on retention of provitamin A and other carotenoids in two biofortified maize genotypes (OPVI and OPVII). Grain was stored at ambient conditions for eight months in PICS bags with and without an O_2 scavenger, (PICS-oxy) and (PICS-noxy), respectively, or in common polypropylene woven bags. After 4 months of storage carotenoid content was significantly higher ($p<0.05$) in PICS-oxy compared to PICS-noxy and woven bags demonstrating the importance of entrapped

oxygen on maize carotenoid degradation. Furthermore, differences in carotenoid stability between maize genotypes were observed with OPVI having higher retention than OPVII. After 8 months, carotenoid retention remained dependent on storage bag and genotype with retention being greater in PICS-oxy and PICS-noxy compared to woven bags. Overall, oxygen content and genotype were determining factors in the effectiveness of PICS to mitigate carotenoid degradation during post-harvest storage of maize.

Key words. Carotenoid; Biofortified orange maize; Provitamin A; PICS bags; Post-harvest storage.

2.2 Introduction

High carotenoid biofortified orange maize (*Zea mays* L.) is promoted in developing countries as a strategy to address vitamin A deficiency through the improvement of the micronutrient density of staple crops (Gannon et al., 2014; Palmer et al., 2016). Carotenoids are fat-soluble plant pigments that can be divided into carotenes and xanthophylls. Carotenoids commonly found in maize are lutein, zeaxanthin, *β*-carotene, *α*-carotene and *β*-cryptoxanthin (Li et al., 2007). Those with unsubstituted *β*-ring end groups (*β*-carotene, *α*-carotene and *β*-cryptoxanthin) maintain provitamin A activity and can be converted to vitamin A (retinol) in humans through the action of intestinal or hepatic *15,15'-β*-carotene oxygenase (Biesalski et al., 2007). The major xanthophylls in maize, lutein and zeaxanthin, do not have provitamin A activity, yet are also important to human health since they accumulate in the macular region of the human retina where they play a role in eye and neuronal development as well as in protection against age-related macular degeneration (Johnson, 2014) and have been associated with improved cognition function in adults (Johnson et al., 2013; Ajana et al., 2018).

Substantial efforts have been made in the development of new varieties of biofortified orange maize with carotenoid levels suitable to impact health in developing countries. Efficacy studies in sub-Saharan Africa have reported the effectiveness of biofortified orange maize in increasing total body vitamin A reserves (Gannon et al., 2014). Efforts to foster translation of these promising grains to at-risk populations have grown through cultivation and adoption of biofortified orange maize in target countries such as Zambia where atleast 126,000 households

had been reached in 2015 (Bouis and Saltzman, 2017; Taleon et al., 2017). Regional interest in this product has increased in various countries that have now released biofortified orange maize and evaluation of biofortified orange maize is on going (http://www.harvestplus.org/knowledge-market/publications).

Currently, the target for maize vitamin A biofortification is 15 μg provitamin A carotenoids (pVAC) per gram of maize in order to provide 50% of the estimated average daily requirement (EAR) for children and pregnant women (Taleon et al., 2017). This relatively high value accounts for expected losses in pVAC through the value chain, including post-harvest storage and cooking (Taleon et al., 2017). In fact, pVAC losses during postharvest storage were observed to be higher than in-home preparation of two biofortified orange maize genotypes released in Zambia (Mugode et al., 2014).

Structurally, carotenoids are highly electron dense and highly conjugated structures that are prone to isomerization and oxidation during storage and processing. Oxidation and isomerization reactions, common in food processing, lead to formation of *cis*-isomers, loss of provitamin A activity and altered bioavailability (Anan et al., 2005). Losses of carotenoids have been observed to be greater in milled products than intact grains when stored under similar conditions (Ortiz et al., 2018; Taleon et al., 2017). These studies highlight temperature, humidity and oxygen exposure as main drivers of carotenoid losses in biofortified orange maize.

Reduction of post-harvest carotenoid losses in biofortified orange maize is needed to facilitate success of ongoing efforts to enhance carotenoid content and positively impact health in developing nations. Considering the main factors described above, implementation of appropriate storage conditions during post-harvest that can moderate key effectors of carotenoid degradation including oxygen and humidity is critical. The Purdue Improved Crop Storage (PICS) System is currently promoted in Africa to reduce post-harvest storage losses of grains due to pests (Murdock and Baoua, 2014). PICS bags have a two high density polyethylene (HDPE) liners (80 µm) inserted inside an outer woven polypropylene sack. The mechanism of control is a bio-generated modified atmosphere based on reduction in oxygen and buildup of carbon dioxide through grain respiration and other biological activities inside the PICS bags. When the oxygen level becomes sufficiently low, insects die of desiccation (Njoroge et al., 2014). To date, only one study (Taleon et al., 2017), has evaluated the use of PICS bags in storing biofortified orange

maize. In this study, retention of pVAC was modestly yet significantly higher in PICS bags (57.2%) compared to common woven bags (51.4%). However, it was not clear the extent to which changes in oxygen and humidity over storage drove these effects. In order to xpand on these findings, the stability of two biofortified orange maize genotyopes stored under similar conditions (relative humidity, temperature, moisture) in PICS bags and woven bags with and without oxygen scavengers was investigated. We hypothesized that maize stored in PICS bags would have higher retention of carotenoids than those stored in woven bags and that by reducing oxygen levels inside the PICS bags, carotenoid retention would be further improved during postharvest storage.

2.3 Materials and methods

2.3.1 Standards and Solvents.

Solvents including acetone, ethyl acetate, methanol (J. T. Baker, Phillipsburg, NJ, USA), methyl *tert*-butyl ether (MTBE) (Sigma-Aldrich, St. Louis, MO, USA) were all certified HPLC grade with >99.9% purity. Ammonium acetate (1.0 M) solution for chromatography was prepared using double distilled water and adjusted to pH 4.6 with glacial acetic acid. Authentic carotenoid standards including lutein, *ß*-carotene, *ß*-cryptoxanthin, *ß*-*apo*-8'-carotenal (Sigma-Aldrich), zeaxanthin (IndoFine, Hillsborough, NJ, USA), *α*-carotene, *α*-cryptoxanthin (CaroteneNature, Lupsingen, Switzerland) were used.

2.3.2 Study design

A 2 x 3 factorial design was used to assess the effect of two biofortified maize genotypes, named open pollinated variety 1 (OPVI) and open-pollinated variety 2 (OPVII), and three storage bag materials (PICS-oxy: with oxygen scavenger, PICS-noxy: without oxygen scaengers) and woven on stability of carotenoids during post-harvest storage. OPVI and OPVII genotypes have an orange colored flinty endosperm and their genetic nature has been previously described (Ortiz et al., 2016). Maize was grown at the Purdue Agronomy Center for Research and Education (ACRE) in West Lafayette, Indiana during the 2016 crop season.

2.3.3 Maize Package and Storage

After harvest, the maize was dried to approximately 8.5% moisture content and immediately packed into PICS bags and woven bags. Representative samples were taken for determination of initial carotenoid content. The first treatment was PICS bags with three Oxy-Sorb oxygen scavenger sachets (Silica Gel Products, Remuera, Auckland, New Zealand) enclosed (PICS-oxy). Each sachet had oxygen scavenging capacity of 2000 cc giving a scavenging capacity of 6000 cc in each bag. This scavenging capacity was enough to lower oxygen levels to below 5% in a 50 kg bag. The second treatment was PICS bags without the oxygen scavenger (PICS-noxy). The third treatment was single layer polypropylene woven bags (Woven). Sampling was done at 0, 2, 4 and 8 months. There were two replicates per genotype, for each treatment and for sampling time, giving a total of 48 bags (50 kg each). The bags were tied with zip tiers starting with the inner layer. The middle and the outer layers were separately tied in manner that helped to ensure that little to no air was trapped inside the headspace of the bags. During time of closing, data loggers (Lascar Electronics, Inc, PA, US) were enclosed inside bags. These loggers were used to record temperature and relative humidity inside the bags and were removed at the time of opening the bags. All bags were stored in the same location with controlled temperature ($29 \pm 1.0\ ^{o}C$) and humidity ($30 \pm 2.0\%$) at the time of storage.

2.3.4 Sampling procedures

Before the bags were opened, a Pac Check MOCON handheld Gas Analyzer needle (Mocon, Minneapolis, MN) was inserted inside the bag to measure internal carbon dioxide and oxygen. Three measurements were taken at different locations for each bag. After measurement, the bags were opened and the biofortified maize was thoroughly mixed before sampling. Immediately after sampling, the maize kernels were stored at -80^{o}C until milling using Foss Tecator 1093 Cyclotec mill (Hoganas, Sweden) and passed through <0.5mm sieve after which carotenoid analysis was performed. In all cases milling was performed within one week of sampling. After milling, samples were taken for carotenoid quantification by Liquid Chromatography (LC).

2.3.5 Carotenoid Extraction

Maize carotenoids were extracted as previously reported (Ortiz et al., 2016). Briefly, ~500 mg of milled grain samples was spiked with 100 μl of *β-apo-8*-carotenal as internal standard. Spiked samples were extracted with 5 mL of chilled acetone (2x) followed by 2 mL of MTBE (2x). The MTBE and acetone fractions were combined and dried under a stream of nitrogen. Prior to LC analysis, dried carotenoids were resolubilized in 2 ml of 1:1 ethyl acetate:methanol filtered through a 0.45 μm PTFE filter and analyzed immediately by LC. Extraction recovery of this method was determined from recovery of the internal standard and found to be 95.3± 3.6%.

2.3.6 LC analysis

Carotenoid separation was carried out on YMC C30 3µm 2.0 mm × 150 mm column, with a YMC carotenoid guard column (2.0 x 23mm) (Agilent Technologies, Santa Clara, CA) in a Hewlett-Packard 1090 HPLC equipped with a Diode Array Detector scanning at 450 nm. Samples were eluted at 0.37 ml/min under the gradient conditions described by Kean et al. (2008). Carotenoids peaks were identified by co-chromatography with authentic *all-trans*-carotenoid standards and comparison with spectral information from previous separations (Kean et al., 2008). Quantitation was completed using a seven point response curve constructed with authentic carotenoid standards in the range of 0.01 to 8.0 µM. Representative chromatogram of biofortified maize can be seen in Supplemental Fig 1.

2.3.7 Data Analysis

Carotenoid contents calculated by external standard curves were expressed as μg/g dry weight and expressed as mean ± standard error of the mean from minimum of n=4 determinations. Data were analyzed by running ANOVA on SAS 94 version (SAS Institute Inc, NC) to determine significant differences between treatment means (PICS-oxy, PICS-noxy, Woven bags) for each genotype. Carotenoid retention (%) was calculated by comparing individual and total carotenoid content at each time point relative to the initial content (t=0). Interaction effects between genotypes and storage bag were determined after 4 and 8 month storage period. Significant differences were determined using the Tukey post hoc test ($p<0.05$).

Pearson's correlation coefficients were generated to quantify the level of association between carotenoid content and humidity.

2.4 Results and discussion

2.4.1 Initial carotenoid content in maize genotypes

Initial total carotenoid content was found to be significantly higher in OPVI compared to OPVII while initial pVACs were similar between the two genotype (Table 2.1). Major carotenoids identified in both genotypes were xanthophylls (zeaxanthin > lutein > *β*-cryptoxanthin > *α*-cryptoxanthin) consisent with previous reports for most maize genotypes including biofortified maize (Mugode et al., 2014; Ortiz et al., 2016). OPVI had higher content of zeaxanthin and lower content of lutein compared to OPVII with similar content reported by previous studies on same genotype (Ortiz et al., 2016). *β*-carotene content was higher in 2012 (4.9 µg/g DW) compared to 2016 (2.5 µg/g DW) . These differences in carotenoid content for OPVI can be explained by the difference in growing season as variations in carotenoid content within the same genotype can be expected between different growing seasons as previously reported (Griffith et al., 2007; Rodroquez-Amaya, 2003). All-*trans*-*β*-carotene was significantly higher ($p<0.05$) in OPVII than in OPVI while *cis*-isomers of *β*-carotene were similar across both genotypes (Table 2.1).

2.4.2 Temperature and relative humidity in storage room and storage bags

The temperature range during the 8 month storage study was related to normal seasonal shifts in West Lafayette, IN, USA (Figure 2.1). During the first four months (December 2016-April 2017), the average temperature inside the storage room was 29.3 ± 1.0 ^{0}C and this was same temperature recorded inside all the bags, Similarly, during the last four months (May 2017-August 2017) the average room and bag temperature decreased to 23.2 ±2.0 ^{0}C. This was directly related to environmental controls in the storage site (i.e. heating in winter and cooling in summer months).

Mean relative humidity (RH) for OPVI was higher (PICS-oxy ~36% and PICS-noxy ~35%) that RH for OPVII (PICS-oxy ~29% and PICS-noxy ~31%) during the first four months (Figures. 2.1A and 2.1B). No apparent affect of placing oxygen scavengers in PICS bags on RH

was observed. RH in PICS bags remained constant because of physical barrier between inside bag and outer environment. The ability of PICS bags to maintain RH has been previously reported (Njoroge et al., 2014; Williams et al., 2017; Mutungi et al., 2014). However, report exists where RH in PICS bags increased dependent on initial moisture content of the grains (Ng'ng'a et al., 2016). Grains with higher moisture increased internal RH more than grains with low moisture. The increase was independent of atmospheric RH. No reasons were given for the increase in RH inside the bags but we speculate that increased physiological activity of grains, related to insect and molds, are likely responsible for this result. During storage of grains, insects and molds use oxygen to oxidize glucose and release carbon dioxide and vapor during respiration (Murdock and Baoua, 2014). The moisture produced may partly explain the increase in RH in PICS bags over time.

In contrast to PICS, RH of OPVI in woven bags ranged from ~ 29%, from 0-4 months and then increased to 58.8% at 8 months (Figure 2.1A). This sharp increase over the last 4 months coinciding with the increase in the storage room RH at the same time period (Fig. 2.1C). Similar patterns were observed for RH of OPVII (Figure 2.1B). Temperature and RH in woven bags were the same as those in the storage room (Figure 2.1C). Although RH did not change during storage in PICS bags for both genotypes it remained modestly higher in OPVI than for OPVII (~3.5% difference). The modest differences might be due to physiological or structural differences of the grain from these genotypes. For example, OPVI is flintier than OPVII, which may affect moisture gain and loss during storage. Overall, these data suggest that RH inside PICS bags is generally stable during storage and similar between maize genotypes.

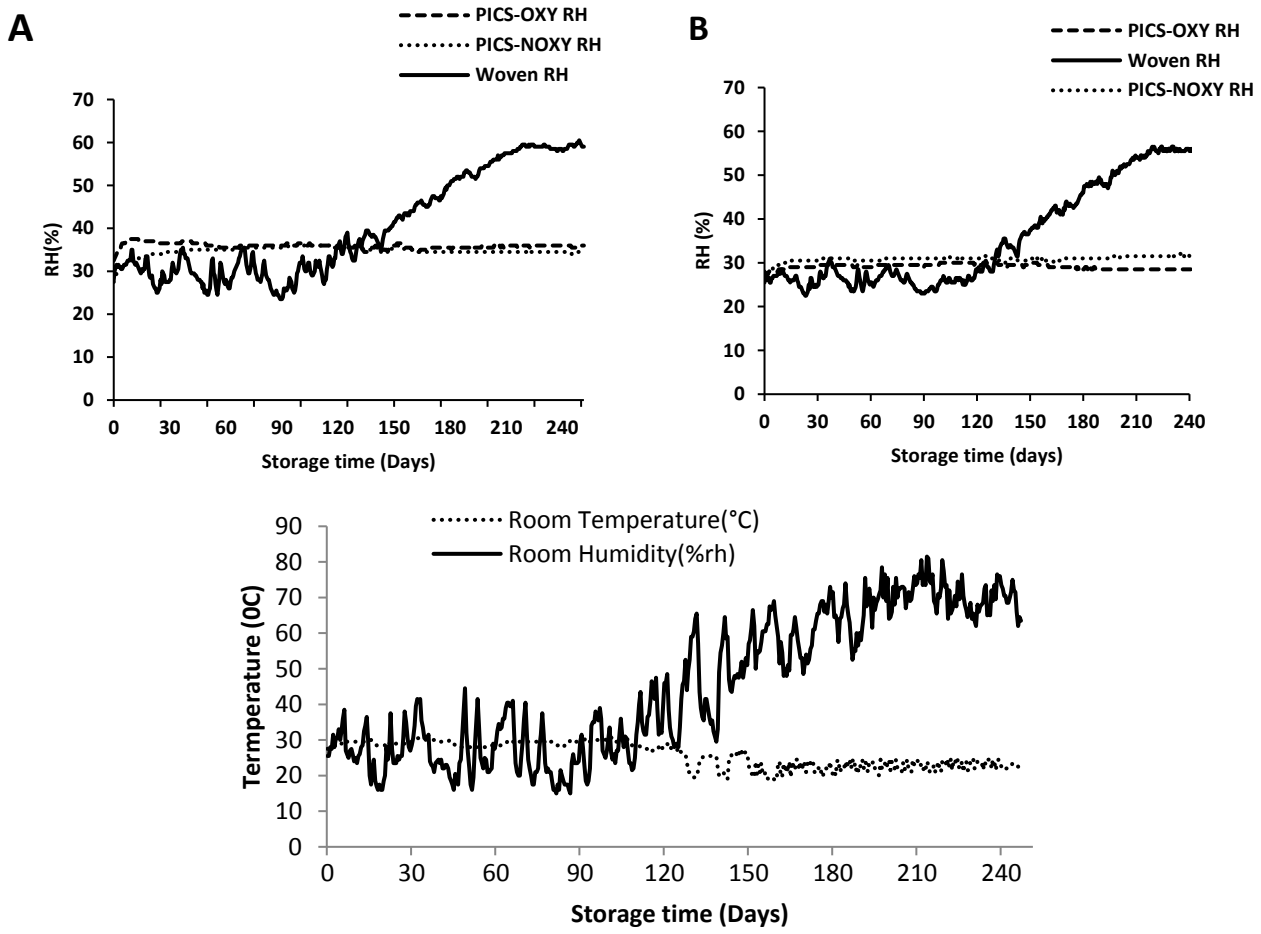

Figure 2.1. Changes in relative humidity (RH) inside PICS bags with scavenger (PICS-oxy), PICS bags without scavenger (PICS-noxy) and woven bags storing OPVI (A) and OPVII (B) maize genotypes for 8 months at Purdue University. Changes in RH and temperature inside storage room (C) during the study period (December 2016-August 2017)

2.4.3 Changes in grain moisture and oxygen levels inside storage bags

The initial moisture of the grain was 8.7± 0.7% for OPVI and 8.4 ± 0.6% for OPVII. After 8 months storage, the moisture level did not change significantly (data not shown). Some studies have reported a decrease (Mutungi et al., 2014; Vales et al., 2014), an increase (Vales et al., 2014), or no change (Ng'ang'a et al., 2016) in moisture content after storing maize grains in PICS bags.

A decrease in oxygen after 15 days was observed in PICS bags containing oxygen scavengers (PICS-oxy) from ~20.3% to 7.3% and 3.0% for OPVI and OPVII, respectively (Figs. 2A and 2B). The difference in reduction of oxygen between bags containing OPVI and OPVII

cannot be attributed only to the presence of oxygen scavengers as we used same scavenging power in all bags. It is plausible that difference in physiological activity between these two genotypes may explain this observation. The physiological activity of OPVII relative to OPVI was not investigated but could differ. In any case, large reductions were observed in oxygen content for both gentypes relative to reduction in PICS-noxy which did not reduce below 18%. This is similar to levels reported by Njoroge et al. (2014) in maize without oxygen scavenger and points to the effectiveness of this scavenger. This inability to reduce oxygen without scavengers in these experiments could be due to several reasons. First the maize used did not contain significant levels of pests that would actively deplete oxygen inside the bags. Physiological activity (i.e. respiration) of the maize grain is therefore the the main driver of oxygen consumption in PICS-noxy system. Grain respiration alone could not reduce the oxygen inside the bags to below 10% as reported by Njoroge et al (2014) due to presumed low respiration rate of the grains at low moisture levels. A higher moisture level of 13-13.5% is recommended for long-term storage (Ng'ang'a et al., 2016), and potentially making maize more biologically active than maize used in this study. Interestingly, during a preliminary study when moisture content was 11.5%, OPVII reduced oxygen inside the PICS bag to 16.5% after 4 months storage (Supplemental Figure 2), a level not achieved after 8 months in the current study, supporting the importance of moisture to physiological activity of stored grains.

As expected, oxygen remained higher in PICS-noxy and woven bags than PIC-oxy bags for the first four months (Figs. 2.2A and 2.2B). Despite a decrease in oxygen in PICS-oxy, we did not detect carbon dioxide inside the bags which suggests that oxygen scavengers, rather than respiration of the grains, were responsible for the decrease in oxygen. However, we detected a small proportion of carbon dioxide in the PICS-noxy. After 15 days, oxygen started to increase inside PICS-oxy until it equaled the oxygen level in PICS-noxy at 4 months. The decrease and then increase in oxygen in PICS bags during storage has been previously observed (Vales et al., 2014), as PICS bags are not a perfect hermetic seal (Mutungi et al., 2014; Ng'ang'a et al., 2016). With oxygen levels reduced by the scavenger initially a differential steep in oxygen pressure between internal and external (room) environment of the PICS bags was created and therefore oxygen likely may have diffused back into the PICS bag.

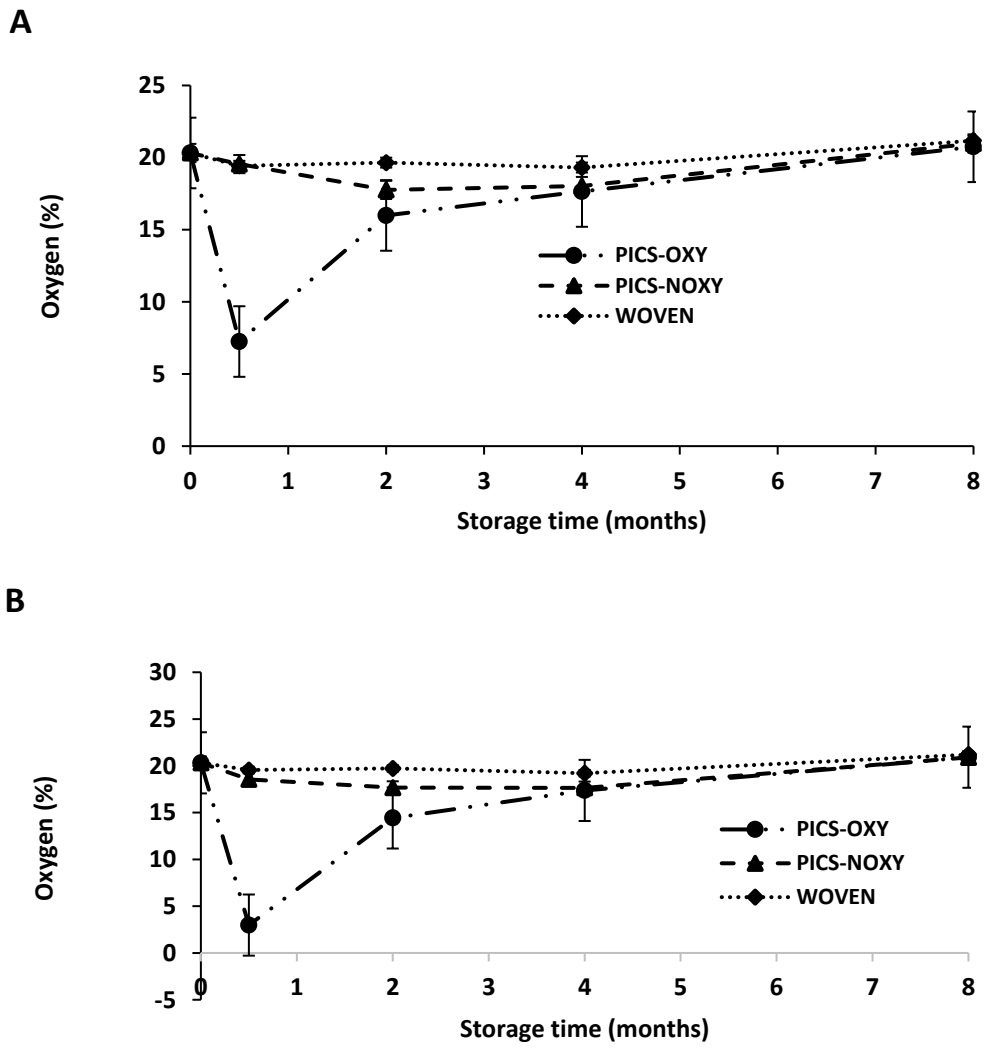

Figure 2.2. Change in oxygen levels inside PICS bags with scavengers (PICS-oxy), PICS bags without scavenger (PIC-noxy) and woven bags storing OPVI (A) and OPVII (B) maize genotypes for 8 months at Purdue University.

2.4.4 Retention of carotenoids through storage

Retention of both pVACs and total carotenoids during storage time was found to be higher in OPVI than OPVII for PICS and woven bags, especially in the first four months of storage. Maize stored in PICS-oxy did maintain significantly higher total carotenoid content ($p<0.05$) compared to PICS-noxy or woven for both genotypes (Table 2.1). After 8 months storage, total carotenoid content in PICS-oxy was significantly higher ($p<0.05$) than in PICS-noxy and woven bags for both genotypes (Table 2.1).

For pVACs, a similar trend was observed across both genotypes with stronger effects in the first 4 months of storage. pVAC content after 4 months in OPVI in PICS-oxy, PICS-noxy and woven bags was 6.8, 4.6, and 5.0 µg/g DW , representing 76, 51 and 56% retention, respectively. pVAC retentions after 4 months for OPVII were 57, 47, and 46% , for PICS-oxy, PICS-noxy and woven bags, respectively (Figure 2.4). After 8 months, pVACs recovery was similar in all storage systems for OPVI and OPVII, respectively, suggesting little impact of PICS bags or oxygen scavenging over longer term storage (Figures. 2.3 and 2.4). However, the current work was only limited to two genotypes with somewhat similar pVAC profiles and additional efforts may be needed to determine the genotype X storage impacts that are optimal.

Retention of xanthophylls was higher in PICS-oxy than PICS-noxy and woven, but extent of enhancement was dependent on genotype (Figures 2.3 and 2.4). Zeaxanthin was found to be more stable than lutein, *α*-cryptoxanthin and ß-cryptoxanthin, which is consistent with previous findings (Ortiz et al., 2016). Content of both lutein and zeaxanthin in OPVI was significantly higher in PICS-oxy than PICS-noxy and woven bags (Table 2.1). In OPVII, the content of lutein (µg/g DW) but not zeaxanthin was significantly higher in PICS-oxy compared to PICS-noxy and woven bags after 4 month storage. Retention of *β*-cryptoxanthin by OPVI was similar in PICS-oxy and PICS-noxy and significantly higher than in the woven bag. Both genotype ($p=0.0024$) and bag type ($p=0.0002$) had significant effect on retention of *β*-cryptoxanthin in both genotypes. Interestingly, there was a significant interaction effect (genotype x bag) on retention of lutein ($p=0.0455$), zeaxanthin ($p=0.0016$) and *β*-cryptoxanthin ($p=0.0334$) after 8 month storage, but not after 4 months.

Maize carotenes were found to be less stable compared to xanthophylls. After 4 months, all-*trans*-*β*-carotene content (µg/g DW) in OPVI in PICS-oxy, PICS-noxy and woven bags were 1.6±0.1 (62%), 1.0±0.1 (39%) and 1.1±0.1 (45%), respectively. OPVII showed similar contents with 1.2±0.1 (45%), 1.1±0.1 (34%) and 1.1±0.1 (34%) in PICS-oxy, PICS-noxy and woven bags, respectively,(Table 2.1) indicating storage system ($p<0.001$) but not genotype ($p = 0.803$) had significant effect on *β*-carotene stability. In all cases PICS-oxy had significantly higher ($p<0.05$) content than PICS-noxy and woven bags. After 8 months, all-*trans*-*β*-carotene content in OPVI was similar in woven bags 1.1±0.1 µg/g DW and PICS-oxy 1.3±0 µg/g DW. Similar lack of effect were observed in OPVII over the full 8 months. However, through all storage periods

assayed, both genotype (p=0.0348) and bag (p=0.0215) had significant effect on content of *β*-carotene. Total *cis-ß*-carotene content was significantly higher in PICS-oxy compared to PICS-noxy or woven bags at 4 month storage but no significant differences were observed after 8 month storage in all the three bags for both genotypes (Table 2.1).

Total *cis-β*-carotene appears to have higher "retention" than all *trans-β*-carotene (Figures 2.3 and 2.4). However this is likely be related more to oxidation of all *trans-β*-carotene than retention of *cis* forms. During storage all-*trans-β*-carotene isomerizes into 15-*cis-ß*-carotene, 13-*cis-ß*-carotene and 9-*cis-ß*-carotene (Rodriquez-Amaya et al., 2011), therefore *β*-carotene isomers increase while all *trans-β*-carotene fraction decrease. Therefore, if the rate of conversion from all-*trans-β*-carotene to its *cis*-isomers is higher than the rate of degradation, *cis*-isomers may accumulate prior to further degradation into epoxides, apocarotenals and apocarotenone (Penicaud et al., 2010). As our analysis was only on a few select time points over an eight month storage period it is likely that such effect could explain these results with *cis*-isomers.

Higher retention of xanthophylls compared to carotenes was expected as stability of these carotenoids to oxidative degradation in maize has been reported to be related to the number of hydroxyl groups in the carotenoid structure with carotenoids having more hydroxyl groups being more stable than those with less or without hydroxyl groups (Ortiz et al., 2016). The presence of additional hydroxyl groups is considered to decrease xanthophyll's reactivity as a radical scavenger and as such would make them less susceptible to oxidative reactions (Xiao et al., 2018), which is consistent with our results (Figure 2.5).

Table 2.1. Carotenoid content (μg/g dry weight) of OPVI and OPVII at the start of experiment and in maize stored in PICS-oxy, PICS-noxy and woven bags during 8 months storage period[1,2,3]

Carotenoids	Genotype	Initial carotenoids	Month 2			Month 4			Month 8		
			PICS-Oxy	PICS-Noxy	Woven	PICS-Oxy	PICS-Noxy	Woven	PICS-Oxy	PICS-Noxy	Woven
Lutein	OPVI	7.21 ± 0.02*	6.76 ± 0.39a	6.04 ±0.10b	5.92 ±0.13b	6.10 ± 0.23a	5.37± 0.23b	5.11± 0.23b	4.50 ± 0.28a	3.97 ±0.15a	4.31 ±0.96a
	OPVII	11.45 ± 0.14*	10.61 ±1.11a	8.88 ±0.45a	9.17 ±0.24a	8.72± 0.46a	7.80 ± 0.70b	7.20 ± 0.40b	6.26 ±0.25a	5.88 ±0.68a	4.66 ±0.31b
Zeaxanthin	OPVI	36.98 ± 0.33*	35.09 ±1.05a	31.07 ±1.54b	30.50 ±1.68b	34.93± 1.51a	29.05 ± 2.24b	27.99 ±1.93b	24.86 ±0.90a	22.81 ±0.75a	19.28 ±0.97b
	OPVII	22.66 ± 0.27*	20.85 ±0.78a	16.69 ±0.65b	19.02 ±0.36c	19.02± 1.20a	17.69 ± 1.52a	16.29 ±1.15a	12.57 ±0.51a	12.13 ±0.50ab	10.94 ±0.52b
α-Cryptoxanthin	OPVI	1.25 ± 0.04	1.13 ±0.09a	0.97 ±0.03a	0.95 ±0.08a	0.97± 0.05a	0.77 ±0.03b	0.81± 0.03b	0.87 ±0.02a	0.70 ±0.16b	0.77 ±0.03b
	OPVII	1.52 ± 0.02	1.47 ±0.25a	1.23 ±0.29a	1.20 ±0.03a	1.13 ± 0.10a	1.11 ± 0.22a	0.92± 0.10a	0.90 ±0.05a	0.87 ±0.08a	0.82 ±0.11a
β-Cryptoxanthin	OPVI	2.76 ± 0.08	2.55 ±0.21a	2.05 ±0.10b	1.99 ±0.08b	2.23 ± 0.05a	1.59± 0.06b	1.65± 0.09b	1.41 ± 0.04a	1.39 ±0.04a	1.16 ±0.10b
	OPVII	2.68 ± 0.02	2.58 ±0.36a	2.01 ±0.48a	1.87 ±0.06a	1.77± 0.18a	1.44± 0.13a	1.38± 0.20a	0.97 ±0.04a	1.07 ±0.03a	1.08 ± 0.23a
cis-β-carotene	OPVI	3.55± 0.15	3.32 ±0.20a	2.86 ±0.13b	2.77 ±0.12b	2.96± 0.12a	1.98 ±0.09b	2.2± 0.11b	1.29 ±0.03a	1.33 ±0.07a	1.21 ±0.13a
	OPVII	3.87± 0.27	3.02 ±0.47a	2.53 ±0.35a	2.31 ±0.49a	2.35± 0.16a	2.11± 0. 09ab	2.05± 0.10b	1.21 ±0.01a	1.21 ±0.01a	1.22 ±0.26a
trans-β-carotene	OPVI	2.55 ± 0.00*	2.10 ±0.15a	1.56 ±0.08b	1.55 ±0.05b	1.58 ±0.08a	0.99± 0.07b	1.14± 0.10b	1.29 ±0.05a	1.18 ±0.04ab	1.06±0.11b
	OPVII	3.19 ± 0.02*	2.90 ±0.35a	2.09 ±0.10b	1.83 ±0.30b	1.45± 0.12a	1.08 ±0.09 b	1.10± 0.13b	1.12 ±0.05a	1.09 ±0.04a	0.98 ±0.21a
Total carotenoids	OPVI	54.30±2.27*	50.80±3.21a	44.58±1.76b	43.56± 1.21b	48.77±2.66a	39.75±2.10b	38.90±1.77b	34.22±0.89a	31.38±1.09bc	27.79±0.35c
	OPVII	45.37±1.44*	41.43±2.28a	33.43±1.51b	35.40±1.01b	34.44±1.21a	31.23±1.16bc	28.94±2.17c	23.03±1.19a	22.25±0.77a	19.70±0.47b
Total pVAC	OPVI	8.86 ± 0.92	7.97 ± 0.87a	6.47 ± 0.56b	6.31 ± 0.89b	6.77 ± 0.47a	4.56 ± 0.23b	5.02 ± 0.14b	3.99 ± 0.10a	3.90 ± 0.05a	3.43 ± 0.12a
	OPVII	9.74 ± 1.01	8.50 ± 1.11a	6.63 ± 0.34b	6.03 ± 0.67b	5.57 ± 0.11a	4.68 ± 0.11b	4.53 ± 0.09b	3.30 ± 0.08a	3.37 ± 0.02a	3.28 ± 0.15a

[1]Data are expressed as mean ± SD (n=4)

[2]) For each storage time, means with different letters within the same row are significantly different according to the Tukey's test ($p<0.05$). cis-β-carotene is the sum of 15-cis- β-carotene, 13-cis-β-carotene and 9-cis-β-carotene; pVAC is the sum of β-cryptoxanthin, cis-β-carotene, trans-β-carotene.

[3] presence of * indicatesinitial carotenoids are significantly different between OPVI and OPVII , Tukey's test ($p<0.05$).

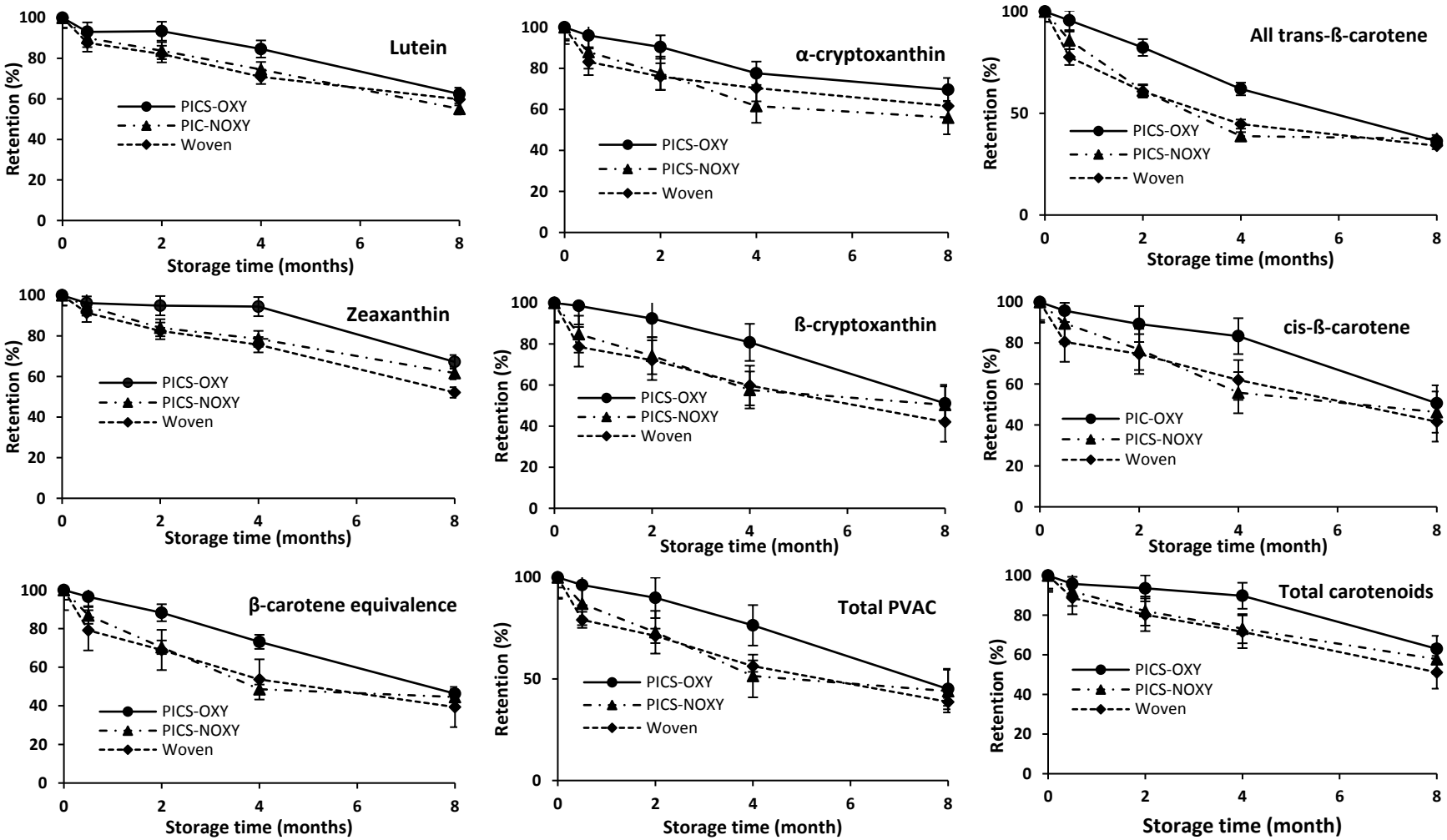

Figure 2.3. Carotenoid rentention in OPVI stored for 8 months. *cis-β*-carotene = sum of 15-*cis*- *β*-carotene , 13-*cis*-*β*-carotene and 9-*cis*-*β*-carotene. Provitamin A in *β*-carotene equivalents = all-trans-*β*-carotene + (*β*-cryptoxanthin + *cis*-*β*-carotene)/2. Total PVAC = sum of all-trans-*β*-carotene, cis-*β*-carotene and *β*-cryptoxanthin). Each point is an average of n = 4 replicates.

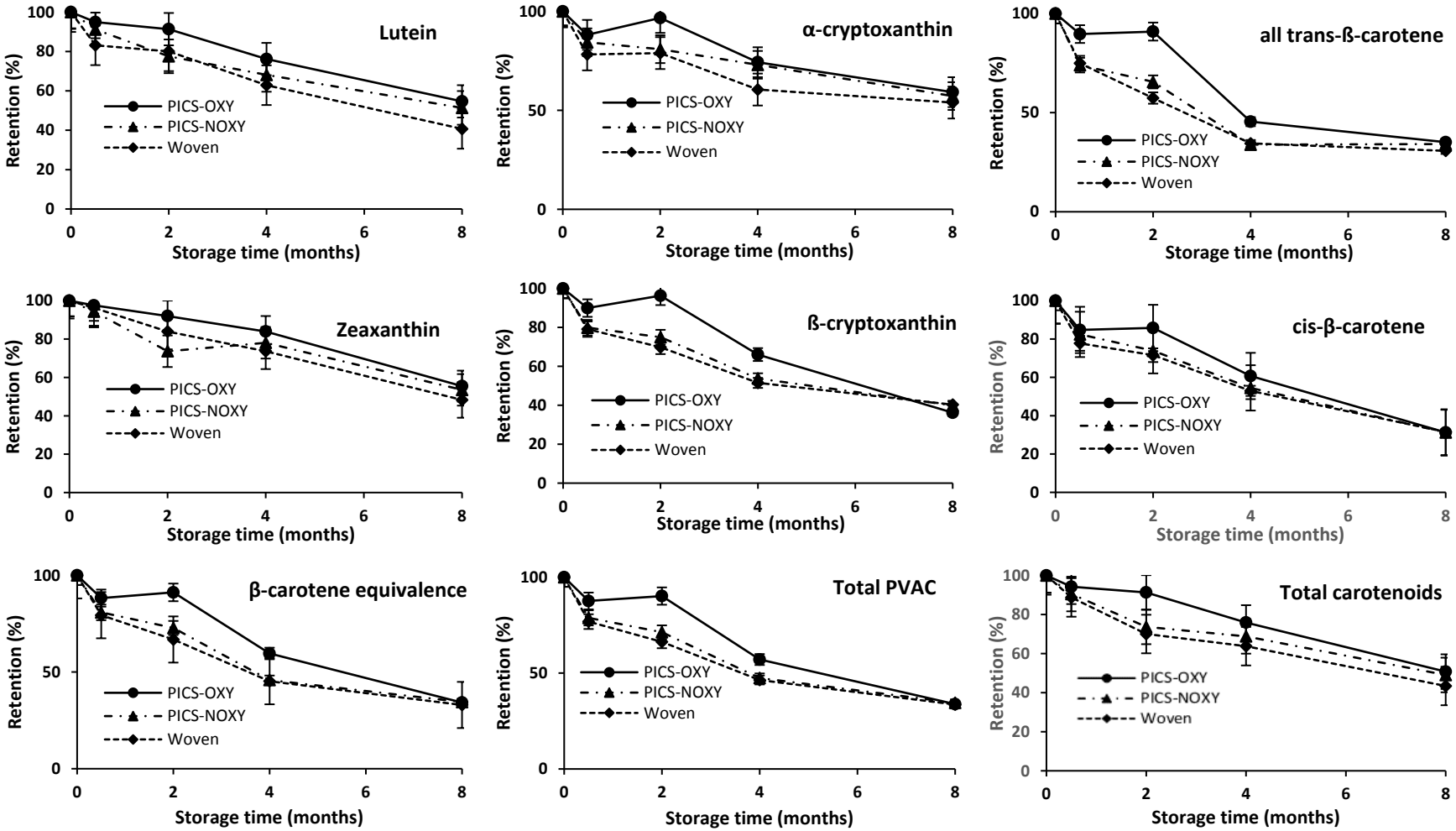

Figure 2.4. Carotenoid retention in OPVII stored for 8 months. cis-β-carotene = sum of 15-cis- β-carotene , 13-cis-β-carotene and 9-cis-β-carotene . Provitamin A in β-carotene equivalents = all trans- β-carotene + (β-cryptoxanthin + cis-β-carotene)/2. Total PVAC = sum of all-trans-β-carotene, cis-β-carotene and β-cryptoxanthin). Each point is an average of n = 4 replicates.

Table 2.2. Pearson correlation coefficients between carotenoid content and storage humidity during storage in PICS-oxy, PICS-noxy and woven bags during 8 months storage period[4,5,6].

Factor		pVAC			Lutein			Zeaxanthin			Total Carotenoid		
		PICS-oxy	**PICS-noxy**	**Woven**	**PICS-oxy**	**PICS-noxy**	**Woven**	**PICS-oxy**	**PICS-noxy**	**Woven**	**PICS-oxy**	**PICS-noxy**	**Woven**
bRH	OPVI	-0.281 (0.647)	-0.859 (0.062)	-0.640 (0.245)	-0.332 (0.585)	-0.696 (0.192)	-0.645 (0.240)	-0.236 (0.703)	-0.742 (0.151)	(-0.795 (0.108)	-0.281 (0.647)	-0.774 (0.124)	-0.745 (0.148)
	OPVII	0.450 (0.391)	0.289 (0.636)	-0.519 (0.370)	0.517 (0.372)	0.170 (0.785)	-0.687 (0.120)	0.437 (0.462)	0.133 (0.831)	-0.759 (0.137)	0.437 (0.462)	0.133 (0.831)	-0.759 (0.137)
rRH	OPVI	**-0.897 (0.039)**	-0.604 (0.281)	-0.682 (0.204)	**-0.904 (0.035)**	-0.801 (0.104)	-0.688 (0.199)	**-0.966 (0.008)**	-0.787 (0.113)	-0.828 (0.084)	**-0.967 (0.015)**	-0.750 (0.144)	-0.781 (0.118)
	OPVII	-0.792 (0.110)	-0.644 (0.241)	-0.617 (0.268)	-0.854 (0.065)	-0.727 (-.164)	-0.772 (0.126)	**-0.922 (0.026)**	-0.757 (0.138)	-0.823 (0.087)	-0.873 (0.053)	-0.727 (0.164)	-0.758 (0.138)

[4]Numbers in parethesis are p-values.
[5]Significant differences when $p<0.05$ or in bold.
[6]Abbreviation; bRH ; relative humidity inside storage bag, rRH; relative humididty inside storage room.

Pearson's correlation coefficients (*r*) were calculated between carotenoid content, bag RH and storage room RH. RH in bag and room were both negatively correlated with carotenoid levels over storage (Table 2.2). The correlation coefficients were lower for bag RH than room RH. Correlation coefficients for room RH were high and significant for pVACs (r =-0.897, p =0.039), lutein (r = -0.905, p = 0.035) zeaxanthin (r = -0.966, p = 0.008) and total carotenoids (r = -0.967, p=0.015) for OPVI and zeaxanthin (r =-0.922, p =0.026) for OPVII in PICS-oxy bags. Room RH was highly and significantly correlated (r = 0.999, p<0.001) with woven bag RH but not PICS bag RH. Generally, this observation may suggest that room RH had more effect on carotenoid degradation than bag RH and that room RH likely determined the RH in woven bags but not in PICS bags.

The differences in stability of carotenoids between the two genotypes might be explained, among other reasons, by differences in kernel porosity, surface area, kernel density and initial total carotenoid content (Ortiz et al., 2016; Weber 1987). OPVI has a flintier kernel type than OPVII and that may have contributed to the higher stability of carotenoids in OPVI. Dent genotypes, or a little less flinty genotypes like OPVII, are more porous while flinty genotypes are compact and less porous. The porosity increases oxygen circulation and therefore makes carotenoids less stable in dent genotypes (Ortiz et al., 2016). Our findings are consistent with the role of oxygen in degradation of carotenoids and that PICS bags could potentially help reduce the rate of carotenoid degradation. Initial total carotenoid content and level of individual carotenoid species determine carotenoid stability during storage (Weber, 1987). In sweet potatoes, genotypes with lower initial total carotenoid content lost less during storage compared to genotypes with higher contents (Bechoff et al., 2010), while carotenoid species that are more abundant tend to have higher losses than those that are less abundant (Weber, 1987). This observation is supported by our results which shows that OPVII which had higher lutein and all-trans-*β*-carotene content than OPVI had higher losses of lutein (5.2 µg/DW (45%)) and *β*-carotene (2.07 µg/g DW (65%)) than OPVI that had lutein and *β*-carotene losses of 2.7µg/g DW (30%) and 0.57 µg/g DW (22%), respectively. This trend was similar for zeaxanthin. OPVI had higher initial content of zeaxanthin and higher absolute loss of zeaxanthin than OPVII.Unlike individual carotenoids, total carotenoids did not follow the '*high initial-high loss*' trend. OPVI had higher initial total carotenoid content (54.3 µg/g DW) than OPVII (45.4 µg/g DW). However, carotenoid loss was higher in OPVII (22.3 µg/g DW, 49%) than OPVI (20.1 µg/g DW, 37%) in

maize stored in PICS-oxy after 8 months. This trend was similar in all other bags (Table 2.1). This discrepancy indicates that carotenoids degradation is a multifactorial process and cannot be predicted simply based on one factor.

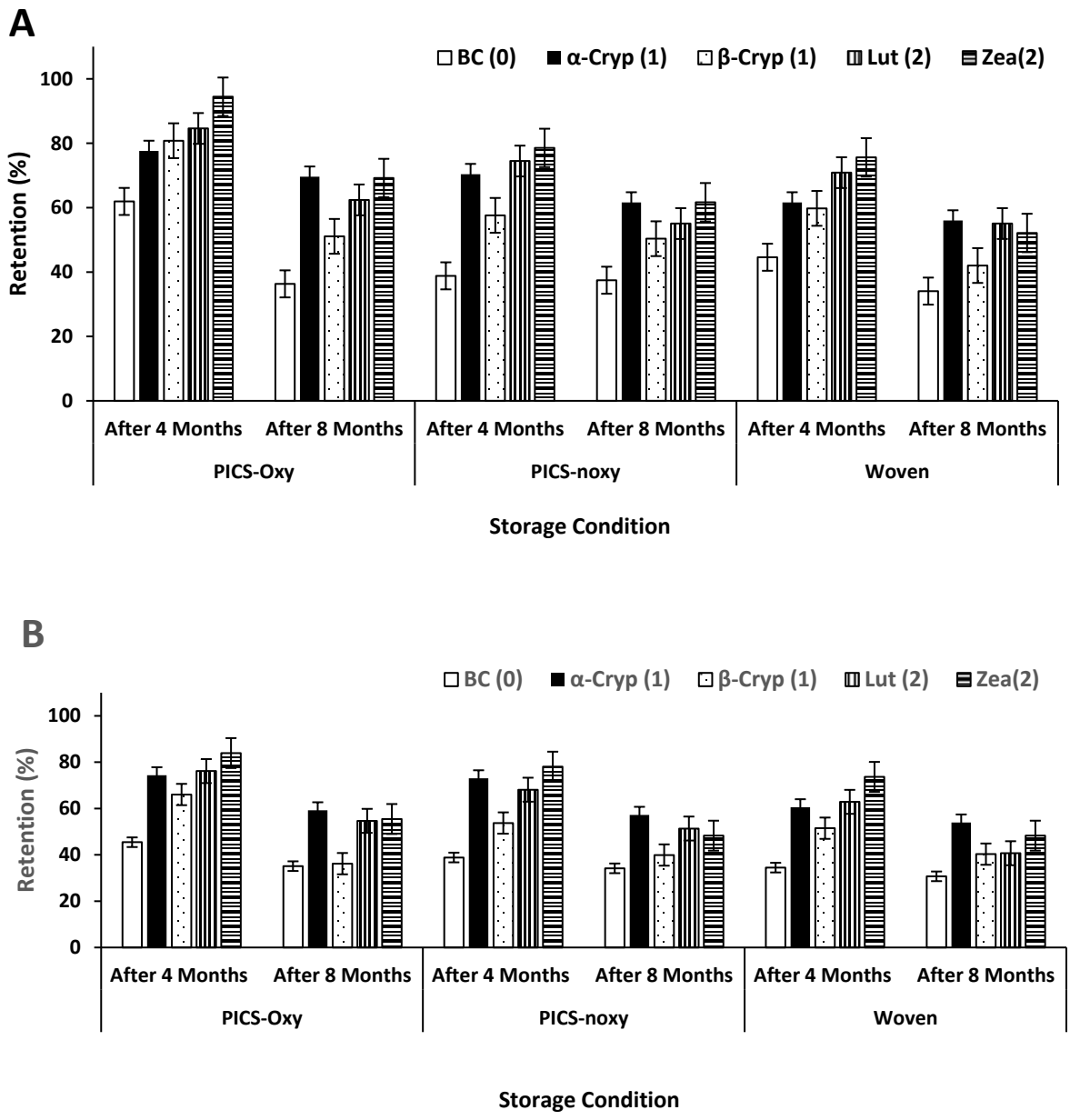

Figure 2.5. Retention of individual carotenoids over 8 months storage for OPVI (A) and OPVII (B). BC = all *trans-β*-carotene, *α*-cryp = *α*-cryptoxanthin, *β*-cryp = *β*-cryptoxanthin, Lut = lutein, Zea = zeaxanthin. Numbers in parentheses indicate number of hydroxyl groups. Each point is an average of n = 4 replicates.

Moth infestation was observed in grain stored in woven bags, while grain in PICS-oxy and PICS-noxy were not damaged or infested by insects (Supplemental Figure 3). This observation is in agreement with the known protective effect of PICS bags from post-harvest pest attack as previously reported (Murdock and Baoua, 2014) and underscores the point that the protective effect of PICS bags against pest does not depend entirely on reducing oxygen inside the bags, but that a physical barrier against pests might be as equally important. Interestingly, insects in the woven bags were found to preferentially consume the germ, which is relatively higher in lipids than the endosperm, leaving the majority of the endosperm intact (Supplemental Figure 3). This observation is of relevance as carotenoids accumulate predominantly in the endosperm (Kean et al., 2008), and any damage to the germ may have only a modest effect on carotenoid content but could impact stability in infested stored grain.

PICS bags appear to be a viable alternative for storing biofortified maize in order to maintain more pVAC nutritional quality, and at the same time provide protection from insects. While PICS bags without scavengers have shown modest effects in reducing carotenoid degradation, sequestering oxygen inside PICS bags through use of an oxygen scavenger seems to have promise in terms of maintianig carotenoid content over modest storage time. However, the applicability of oxygen scavengers may be limited for most households in developing countries and alternative strategies to deliver on modified atmosphere storage (including enhancement for physioplogical activities of grains) should be explored. In fact, reducing oxygen levels during packing and closing of the bags improved carotenoid stability even after the oxygen level rose again. Unfortunately, this protective effect is lost during longer-term storage (6-8 months) suggesting further exploration of factors to enhance the stability of carotenoid in long-term storage. Depending on the nature of the grain, level of insect pest (for initial oxygen drop), duration of storage and extent of grain moisture, PICS storage of biofortified maize could help in maintaining the nutritional quality of high carotenoid biofortified orange maize more than woven bags. While this research is limited by number of genotypes that were used, it does offer insights on an economical way to store biofortified maize to improve retention of nutritional quality in countries where PICS is already commercialized and highly diffused.

CHAPTER 3. EFFECTS OF PURDUE IMPROVED CROP STORAGE (PICS) BAGS ON FLOUR RHEOLOGY AND FUNCTIONALITY

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3.1 Abstract

Success of biofortified maize is dependent, in part, on consumer adoption of these grains and use in traditional food preparation. Assessment of the rheological and functional properties of elite biofortified maize genotypes as a function of post-harvest storage is therefore needed. The impact of post-harvest storage in PICS bags on four functionality and rheological properties for white and two orange biofortifed maize genotypes (OPVI and OPVII) was assessed. Grains were stored for 8 months in PICS-oxy (PICS bags with oxygen scavenger enclosed), PICS-noxy (PICS bags without oxygen scavenger) and traditional polypropylene woven bags. Flour pasting profiles were assessed initially and at 4 and 8 months. After 8 month storage in woven and PICS bags, OPVI and OPVII produced porridges with similar viscosities to initial viscosities regardless of postharvest storage type. White maize viscosities progressively decreased with storage and were significantly lower ($p<0.05$) in woven compared to PICS storage. Sequestration of oxygen (PICS-oxy) had modest but significant effects ($p<0.05$) on key pasting parameters including peak and final viscosities. These results suggest that oxygen sequestration had a critical effect on final flour functionality. DTT treatment partially restored flour pasting profiles suggesting disulfide linkages may modify pasting profiles of flour. Increase in free phenolic acid during storage was related to decrease in porridge viscosities as well as decrease in spectral intensity at both 478cm^{-1} and 2911cm^{-1} suggesting the potential for structural changes induced by storage on starch polymer. While storage in PICS bags does not seem to adversely affect flour functionality it provides an additional potential economic benefit resulting from

requiring proportionally less flour to achieve similar final viscosities as flour from woven bag stored grains.

3.2 Introduction

Over the past 10 years, significant effort has been placed in the development of nutritionally biofortified maize varieties suitable for introduction into Sub-Saharan African markets with a primary focus being on achieving target levels for micronutrients such as provitamin A carotenoids. This has culminated in the introduction of biofortified orange maize genotypes in several areas of Sub-Saharan Africa (Bouis and Saltzman, 2017). While nutritional targets have been the primary focus, successful translation of biofortified maize will also depend on the consumer acceptability of milled maize and finished products generated from these new biofortified genotypes. Interestingly, there remains a general lack of published information on the comparative food functionality of these biofortified maize flours relative to the common white maize varieties used in Africa. With that in mind, a better understanding of the food functionality of biofortified grains and derived products is needed to better understand the product use and foster consumer adoption of these improved grains.

While biofortified maize has been primarily investigated for their provitamin A levels, food functionality of maize is dependent on the macronutrient components including starch, proteins, fats and other non-starch polysaccharides. Starch is the major component of maize comprising up to 71% of the total mass of maize flour (Eckoff and Watson, 2009). Maize starch consists of primarily of amylose (15-30%), a linear polymer, and amylopectin (70-85%), a branched chain polymer (Manek et al., 2012). Functional properties of typical maize flours are dependent on both the amount of starch as well as amylose:amylopectin ratio (Awazuhara et al., 2000). Starch plays a large role in determining the final texture of maize based foods, especially thin and thick porridges commonly consumed as staple foods in Sub-Saharan Africa. Assessment of starch properties in biofortified maize has been limited. Oluba and Oredokun (2018) reported that a Nigerian white genotype had similar starch content as a biofortified yellow maize (BRY 9928 DMR SR) genotype. Interestingly, the biofortified maize was reported to have a significantly lower glycemic index compared to the white maize suggesting that other factors,

including carotenoid content, may contribute to differences in starch digestibility and possibly starch functionality.

In addition to starch functionality in fresh grain, post-harvest storage is also known to induce both physical and chemical changes impacting grain quality and functionality for subsequent food applications. During long-term storage cereals undergo an "aging" process that alters physicochemical, functional and nutritional properties of derived flour (Zhou et al., 2002; Fierens et al., 2015; Setiawan et al., 2010). This includes decreased protein solubility due to protein cross-linking resulting from disulfide linkages (Zhou et al., 2002; Chrastil and Zarins, 1992). Post-harvest storage of maize is known to result in lower protein and starch digestibility (Rehman et al., 2002). Moreover, increases in disulfide linkages during storage inhibit starch granule swelling (Hamaker and Griffin, 1990) which directly affects cooking performance and final texture of traditional thin and thick porridges.. Such changes are critical in determining final consumer attribute and remain dependent on post-harvest storage temperature and time with higher temperature and longer storage time inducing profound changes compared to low temperature and shorter storage time (Paragonski et al., 2014; Fierens et al., 2015).

Of the storage systems considered for implementation of biofortified grains in Sub-Saharan Africa, the Purdue Improved Crop Storage (PICS) bag system is low cost storage bags for grains with the ability to reduced post-harvest losses from insect damage (Njoroge, et al 2014; Murdock and Baoua, 2014; Baoua et al., 2014). As a passive modified atmosphere storage system, PICS systems have also shown some promise in mitigating provitamin A carotenoid losses in biofortified orange maize through modification of oxidative conditions (Taleon et al., 2017; Nkhata et al., 2019). This point is critical considering that post-harvest ageing of grains requires oxygen. High oxygen levels have been reported to accelerate the ageing process (Groot et al 2012) with low oxygen levels slowing down the process. Considering the ability of PICS bags to modify available oxygen, the potential exist for this system to potentially alter post-harvest ageing of grains and ultimate functionality of finished maize flours. With this in mind, the primary objective of this study was to determining effect of PICS post-harvest storage of maize on flour functionality. We hypothesize that sequestering oxygen in PICS bags, by biotic or abiotic means, would slow down ageing process and subsequently alter conventional and biofortified maize flour functionality by altering pasting properties.

3.3 Materials and methods

3.3.1 Materials

Two biofortified orange maize genotypes, OPVI and OPVII, used in this study were grown, dried and stored as previously reported (Nkhata et al., 2019). The genetic nature of these genotypes has been described elsewhere (Ortiz et al., 2016). The white genotype was of OPV type and was grown at same field as biofortified maize genotypes. *1,4* Dithiothreitol (DTT) was purchased from Sigma-Aldrich (St Louis, MO). Methanol, formic acid, ethyl acetate (J. T. Baker, Phillipsburg, NJ, USA), Oxy-sorb scavengers (Silica Gel Products, Remuera, Auckland, New Zealand) were also used. Analytical standards of ferulic acid and *p*-coumaric acid were purchased from Sigma Aldrich (St. Louis, MO, USA).

3.3.2 Study design

A 3 × 3 factorial design was used. Fifty kilogram of maize were sorted and stored in PICS bags with Oxy-Sorb oxygen scavenger enclosed (PICS-oxy), without oxygen scavenger (PICS-noxy) and traditional polypropylene woven (woven) bags for 8 months. These bags were intended to study the degradation of carotenoids in PICS bag and results have been previously reported (Nkhata et al., 2019). In addition to the two biofortified maize genotypes, a third white maize genotype was used to further understand effects of storing maize in PICS bag compared to woven bags. Sampling and analysis was done at 0, 4 and 8 months.

3.3.3 Pasting profiles of maize flours

Pasting properties were determined from maize flours using Rapid Visco Analyzer (RVA) (Newport Scientific RVA-4, Australia). Maize kernels were ground to flour by Foss Tecator 1093 Cyclotec mill (Hoganas, Sweden) and pass through <0.5mm sieve. A 3.5 g aliquot of flour corrected to 14% moisture was suspended in ~25ml deionized water. A programmed heating and cooling was used at 50^{o}C for 1 minute and slowly heated to 95^{o}C at 6 ^{o}C per minute, held at that temperature for 2.7 minutes, before cooling from 95 to 50^{o}C at 6 ^{o}C per minute and holding at that temperature for 2 minutes. Data was generated on Thermocline for Window version 1.2 software.

In order to determine the effect of storage on disulfide linkages formation and pasting properties, a separate aliquot of flour from all sampling intervals were treated with 10μM 1,4

Dithiothreitol (DTT). DTT disrupts disulfide bonds formed during storage (Hamaker and Griffin, 1990) and allowed for comparison of DTT treated, non-DTT treated and initial samples at each storage time (0, 4 and 8 months). Samples were similarly analyzed using RVA as described above.

3.3.4 Extraction and determination of maize phenolics:

Free phenolics were extracted from whole grains samples as previously reported (Li et al., 2016). Briefly 100 mg of flour was extracted three times with 3 mL of 80% methanol containing 0.2% formic acid. Extracts were dried under nitrogen and further extracted three times with ethyl acetate. The ethyl acetate extract was dried under nitrogen and resolubilized in 80% methanol containing 0.2% formic acid for immediate analysis by UPLC-MS as described by (Li et al., 2016)**.**

3.3.5 Determination of structural changes of starch using Raman spectroscopy

Raman spectra of maize flour was obtained on Bruker MultiRAM FT-Raman Spectrometer (Bruker Optics Inc, Billerica, MA) with Nd:YAG laser with sample excitation source at 1064 nm, equipped with nitrogen cooled Germanium diode detector. The flour sample (~4mg) was placed on small aluminum sample cup. A 350mW laser power was used for excitation. For each spectrum an average of 500 scans were performed at a spectral resolution of 4 cm^{-1}over 3500-50 cm^{-1} range. Assignment of Raman spectral information (peak position and corresponding chemical bonds) was based on information reported in the literature and presented in Table 3.2 (Kizil et al., 2002; Almeida et al., 2010; Wang et al., 2015).

3.3.6 Data analyses

Data are reported as mean ± standard deviation from a minimum of triplicate analyses for phenolic analysis and pasting properties. Analysis of pasting profiles included comparison of viscosities in centipoise (cP); peak viscosity (PV), final viscosity (FV), trough (T), breakdown (BD), setback (SB), peak time (PT) and pasting temperature (PTemp) as a function of storage time and storage method for each genotype. Phenolic acids profiles and statistical analysis was conducted by a two-way ANOVA using SAS 9.4 version (SAS Institute Inc, NC) with genotypes (3) and storage systems (3) as factors. Comparisons were made in two different ways; between

bags (PICS-oxy, PICS-noxy, woven) at each storage period and between time points (0, 4, 8 months) followed by Tukey's HSD test (α=0.05) for determination of significant differences.

3.4 Result and Discussion

3.4.1 Comparison of initial pasting profiles of the three genotypes.

Pasting profile key parameters (Figure 3.1) for flour derived from fresh grains of the three genotypes were derived. PV, FV, PT and SB for two biofortified orange maize genotypes were significantly lower than white maize genotypes suggesting initially that the biofortified genotypes do not provide the same ability to build viscosity indicating potential differences in cooking performance as a more standard white cultivar. Particularly important to consider is the differences observed in both PV and FV. PV is the maximum viscosity achieved by the flour slurry during the heating portion of the RVA test (Adedokun and Itiola, 2010). PV is an important parameter during cooking of porridge and/or *nshima* as it determines the ease of cooking and for consumers, is related to the amount of flour used to achieve a desirable product thickness. This parameter is consistently used as an indicator of cooking performance (Adedokun and Itiola, 2010). Similarly, FV is viscosity at the end of the test (Adedokun and Itiola, 2010) and more closely represents the serving viscosity of porridge/*nshima* or texture as would be seen by consumers. Food texture is considered critical to consumer perception as it represents a factor that impact consumer preferences/expectations (Stevens and Winter-Nelson, 2007).

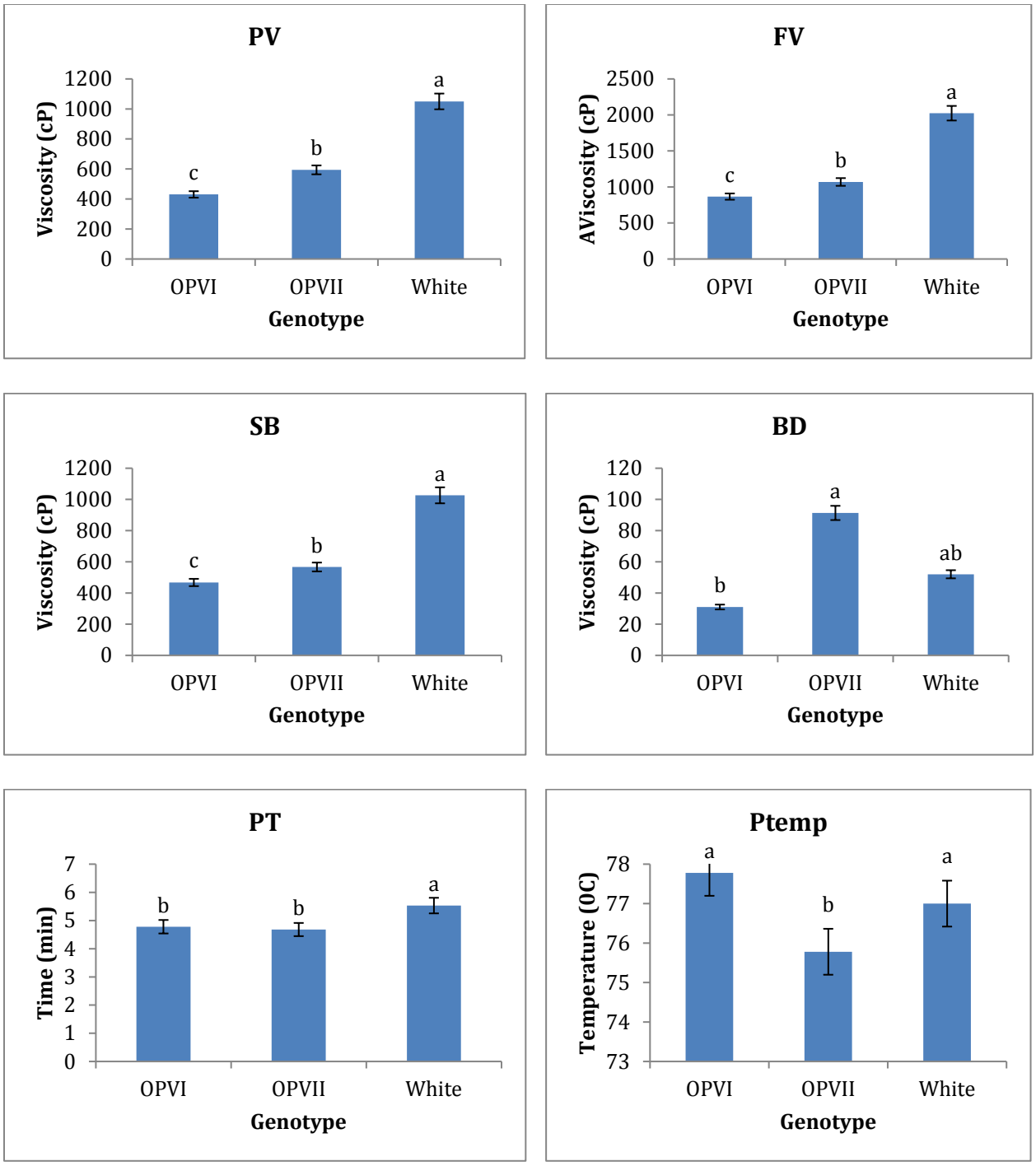

Figure 3.1. Initial pasting profiles of flour derived from fresh grains (before storage) of two biofortified orange maize genotypes (OPVI and OPVII) and one white maize genotype. PV; peak viscosity, FV; final viscosity, SB; setback, BD; breakdown, PT; peak time; PTemp; pasting temperature. Bars with different letters per parameter are significantly different between maize genotypes Turkeys test $p< 0.05$.

3.5 Changes in pasting profiles of flour after storage in PICS and woven bags

Pasting profiles were determined through storage to assess changes in these properties as a function of storage time, storage method and genotypes. PV for OPVI did not change with storage while that of OPVII and white genotype changed depending on storage bag system. Woven bags generally produced flour with significantly lower ($p<0.05$) PV than flour from initial grains and those stored in PICS bags (Figure 3.2). Similarly, FV for all genotypes were significantly lower ($p<0.05$) in woven bags compared to initial grains. In addition, PICS bag stored grains produced porridge with higher FV compared to initial FV (Figure 3.2). These results suggest that storage bag may influence flour pasting profiles differently for these two biofortified maize genotypes. After 8 months the storage effect on PV was dependent on both genotype (G) ($p<0.0001$) and storage bag (B) ($p<0.0001$) with significant G × B interaction effect ($p<0.0001$). Similarly, both genotype ($p<0.0001$) and storage bag ($p = 0.0017$) had significant effect on FV. There was also B × G interaction effect ($p = 0.0016$). The genotype, bag and interaction effects reinforce the notion that changes in pasting properties during storage are complex phenomenon and depend extensively on the nature of the maize, storage method or both.

While changes were expected to occur during storage (Zhou et al., 2003), these results suggest that storage of grains in PICS bags may alter ageing process that normally occurs in grains stored in woven bags. The effect was more profound in white genotype than biofortified orange maize genotypes after 8 months storage (Figure 3.2). Reducing oxygen using a scavenger in PICS-oxy had modest but significant effect on both PV and FV relative to PICS-noxy for OPVII but not OPVI after 8 months storage (Figure 3.2). Moreover, FV was significantly ($P<0.05$) higher in PICS-noxy than woven bags for all genotypes suggesting that both oxygen sequestration and PICS bags had independent effect on FV. While FV for OPVI and OPVII decreased to initial level after 8 month storage, FV for white maize genotype remained significantly lower than initial FV (Figure 3.2).

Several factors may play into the observed alterations of PV and FV in stored maize. Previously, reduction in PV of rice during storage was associated with an increase in polymerization of protein through disulfide linkages (Hamaker and Griffin, 1990) as well as an increase in starch crystallinity (Setiawan et al., 2010). These storage changes lower ability of

starch granule to swell freely before rupturing as the kernel becomes progressively more organized during storage (Zhou et al., 2003).

FV is a function of a numerous factors among which starch retrogradation plays a central role (Alcazar-Alay and Meireles, 2015). While retrogradation is sometimes undesirable for food industries it is an important parameter as it determines the viscosity of the paste (ie porridge or *nshima*) during serving and has significant influence on perceived texture of the porridge. A more viscous porridge/*nshima* is generally preferred as is perceived to be more satiating (Cisse et al., 2018; Marciani et al., 2001; Santangelo et al., 1998; Marciani et al., 2000). Moreover, highly retrograded starch is less digestible (Lovegrove et al., 2017) which can confers other benefits. The ability of PICS bags to maintain higher viscosity relative to woven bags has additional economic implication. Comparatively, less flour would be required to make porridge with exactly the same final viscosity (texture) from grains stored in PICS bags compared to woven bags. Cost savings could be found at the household level using grains stored in PICS bags due to both reduction in post-harvest losses to pest and better performance in product requiring proportionally less flour to achieve acceptable viscosities.

The percentage increase in FV in PICS bags relative to woven bags were; OPVI 28% in PICS-oxy and 16% in PICS-noxy; OPVII 6% in PICS-oxy and -14% in PICS-noxy; White 50% in PICS-noxy. These figures suggest that increase in FV resulting from PICS bags storage is dependent on genotype with white genotype producing largest increase. With the potential to minimize post-harvest losses (Njoroge et al., 2014), improve carotenoid retention (Taleon et al., 2017; Nkhata et al., 2019) and provide a product with improved cooking performance, PICS bags are viable alternative to improve both food and nutrition security in developing countries.

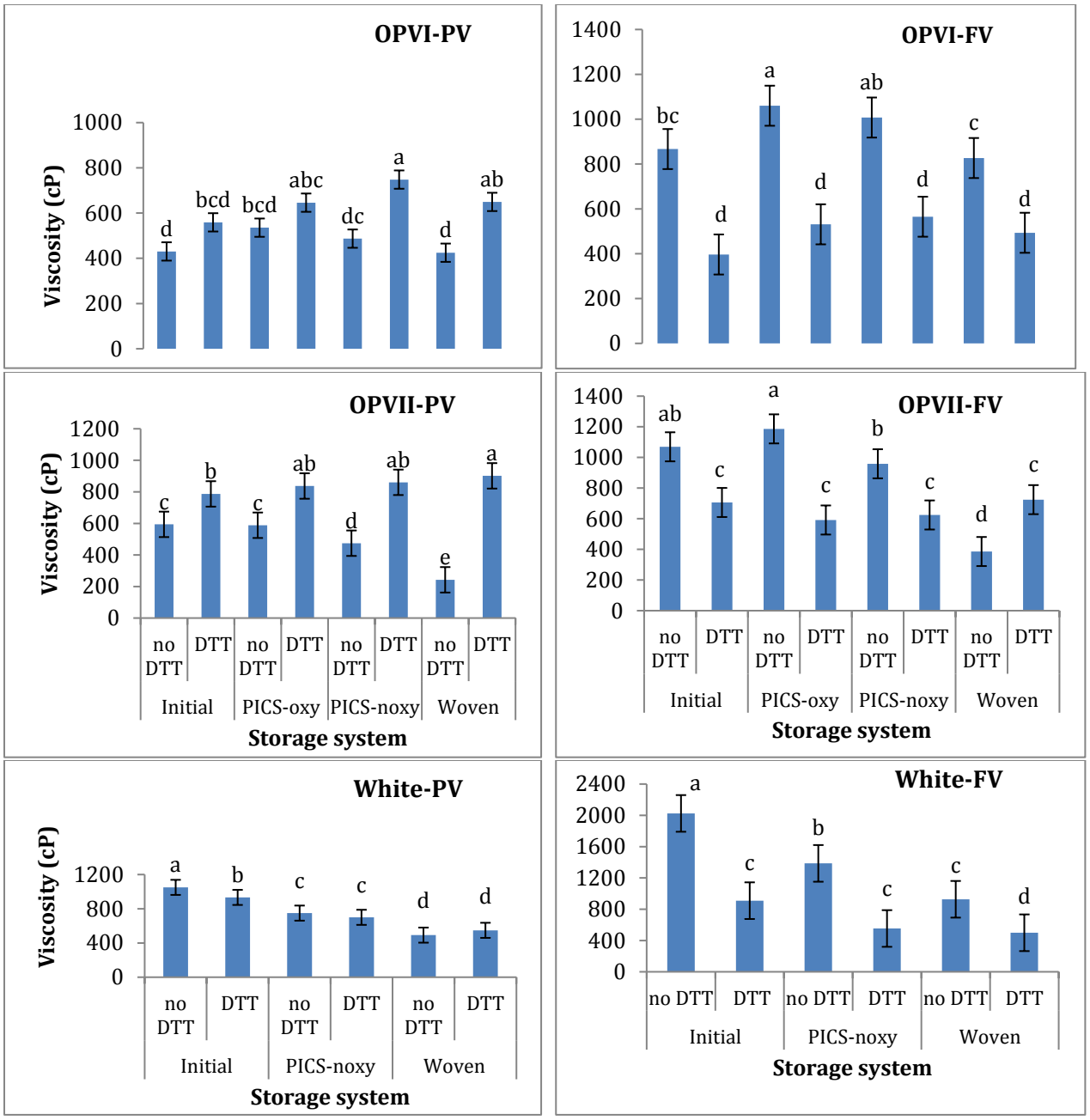

Figure 3.2: PV and FV of flours from initial and stored grains treated with or without DTT after storage for 8 months in PICS-oxy, PICS-noxy and Woven bags. PV; peak viscosity, FV; final viscosity. Bars with different letters for each parameter are significantly different Turkeys test $p < 0.05$.

Other pasting properties were impacted but not consistently by storage. For example, SB did not change significantly for two biofortified maize genotypes but progressively decreased in white maize during 8 month storage to levels significantly lower in woven compared to PICs

(Supplementary table 2). A similar decrease in SB during storage was reported in maize starch extracted from stored maize (Setiawan et al., 2010) and could be related to starch-phenolic acid complexation (Li et al., 2018) which interfered with amylose re-association during retrogradation resulting in a less viscous or softer porridge. Storage time or method did not have significant effects on BD and PTemp across genotypes (Supplemental table 1 and 2). There was a modest but significant increase in PT for OPVI and OPVII but not white maize after 8 month storage relative to initial grains (Supplemental table 2). The increase in PT is indicative of longer cooking time and may indicate either a shift to a more organized starch granule or that starch granules was embedded in non-starch components that limited its swelling (Tester and Morrison, 1990; Paraginski et al., 2014).

3.6 Effect of disrupting disulfide linkages on pasting profiles

As we hypothesized that PICS bag storage may alter ageing-induced properties that include disulfide bond formation, we disrupted disulfide linkages directly by adding 10μM DTT to cooking water to better understand the contribution of proteins to changes in viscosity. DTT is a strong reducing agent that breaks down cross-linked protein structure resulting from disulfide bond (~S-S~) formed between neighboring cysteine amino acids or other sulfur containing amino acid. These bonds are formed during storage of cereal grains. The protein cross-linking forms protein networks that make starch granule hard to swell. Once the disulfide bonds are reduced (~SH) the network is broken and loosened and the viscosity of cooking porridge is increased (Hamaker and Griffin, 1990). Indeed, treatment of samples with DTT significantly increased PV and decreased FV in initial fresh grain samples as well as stored (aged) grains for biofortified orange maize genotypes (Figures 3.2 and 3.33). DTT had no effect on PV of white maize in both woven and PICS-noxy bags but had effect on initial grains (Figure 3.2) indicating that a decrease in PV observed during storage of white maize was not likely due to disulfide linkages but likely other factors. Both PT and PTemp decreased after DTT treatment of all the three genotypes (Supplemental tables 1 and 2). FV for white maize porridge decreased with storage but decreased further when treated with DTT an observation that confirm the role of protein in stabilizing paste or porridge (Figure 3.2).

The increase in PV in initial sample indicates that disulfide linkages might have been present in initial un-aged grains as well. The effect of DTT on increasing PV was higher in stored samples than initial samples indicating more disulfide bond were likely formed during storage (Figure 3.2, Supplemental tables 1 and 2). For example, PV of initial sample for OPVI increased from 430.3 centipoise (cP) to 559.0 cP. Treating 4 months stored samples with DTT increased PV from 602.3 to 880.0cP in PICS-oxy, 557.7cP to 811.0cP in PICS-noxy and 497.3cp to 756.0cP in woven bags (Supplemental table 1). These DTT mediated increases were found to be dependent on bag type ($p<0.05$) and were significantly ($p<0.05$) higher in porridge from stored grains than from initial grains.

It is important to note that treatment of stored grain samples with DTT did not generally restore the viscosity fully to those observed in initial samples that were treated with DTT indicating that apart from disulfide linkages other factors might have contributed to this effect (Supplemental table 1 and 2). This observation was made more clear in experiments with white maize (Figure 3.2). DTT treatment generally leveled the effect of bags in stored samples (Figure 3.2, Supplemental tables 1 and 2) confirming the differences in viscosities observed in bags were due to disulfide linkages.

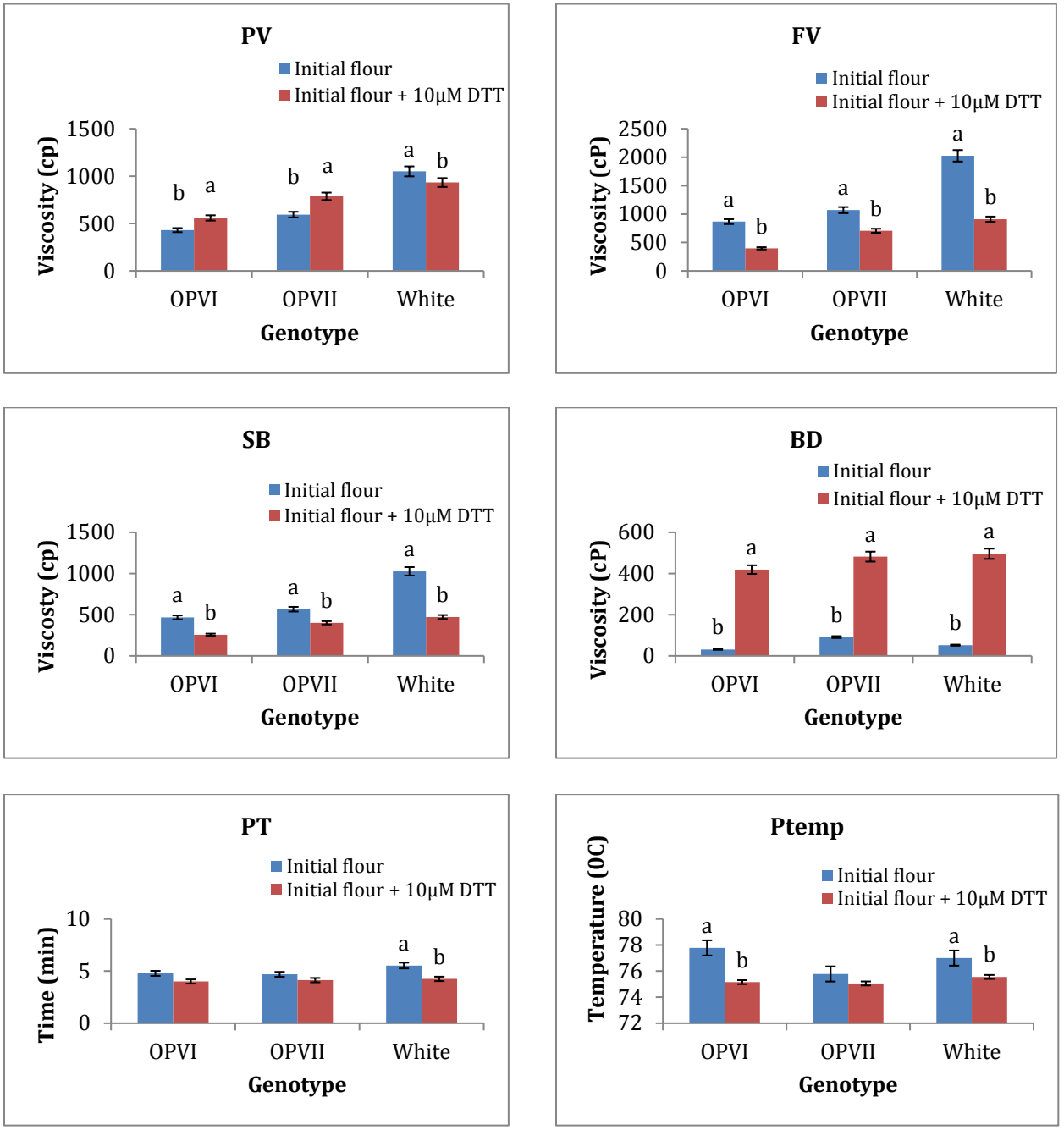

Figure 3.3. Pasting profiles of initial flour with or without 10µM DTT. PV; peak viscosity, FV; final viscosity, SB; setback, BD; breakdown, PT; peak time; PTemp; pasting temperature. Bars with different letters for each genotype are significantly different Turkeys test $p < 0.05$.

DTT treated samples had significantly higher breakdown (Figures 3.3, Supplemental tables 1 and 2) indicating that DTT treated pastes are highly unstable and could easily disintegrate, an observation that confirms importance of disulfide linkages to the stability of paste. The decrease in PT and PTemp in DTT treated samples confirms that disulfide linkages formed during storage makes starch granule hard to swell and rapture and that more time and higher temperature are required to swell and rapture it. These changes may also be related to increased starch crystallinity as significant changes ($p<0.05$) in PT an indicator of increased starch crystallinity, (Tester and Morrison, 1990; Paraginski et al., 2014) was observed in non-DTT treated samples for OPVI and OPVII after 8 month storage (Supplemental table 2). Therefore, these changes are attributed to protein polymerization, potential increased starch crystallinity or effect of non-starch components surrounding the starch granules that limit starch granule swelling (Teo et al., 2000; Kumar and Ali, 1991; Zhou et al., 2003).

3.7 Changes of free phenolic acids during storage in bags

Considering that disruption of disulfide bonding by DTT did not restore all porridge viscosities, especially at 4 month storage assessment of secondary factors including free phenolic release through storage were considered. Presence of free phenolic acids in starch systems have shown the ability to interact directly with starch and form starch-phenolic complexes that have been documented to alter pasting profiles of model starch systems (Li et al., 2018). Ferulic and *p*-coumaric acids are the main phenolic acid present in maize of which ferulic acid is most abundant (Zhou et al., 2002). Complexation of ferulic acid with native amylopectin and potato starch significantly decreased PV, cold paste viscosity and PTemp (Li et al, 2018) through a mechanism involving competition for water molecules between amylose and/or amylopectin and free phenolic acids that limited hydration of starch (Li et al., 2018). It is plausible therefore that a release of free phenolics may in fact be related to the modifications in pasting properties observed.

Interestingly, storage of maize for 8 month did result in an increase of measurable free ferulic and *p*-coumaric acids (Table 3.1) with highest increases in maize stored in woven bags. In OPVI genotype free ferulic acid increased from 31.4±6.7 µg/g at the time of storage to 48.9±5.7 µg/g after 8 month storage in PICS-oxy and to 60.1±7.9 µg/g in woven bags while *p*-coumaric

acid increased from 0.23±0.0 to 0.33±0.1 and 0.6±0.1 µg/g in PICS-oxy and woven bags, respectively. Free ferulic acid also increased in both OPVII and white genotypes with highest increase in woven bag compared to PICS bag (Table 3.1). In OPVII, *p*-coumaric acid did not change in both bags and decreased in PICS-oxy for white genotype. Genotype (G) ($p = 0.0020$) and storage bag (B) ($p = 0.0040$) had significant effect on the release of ferulic acid during storage. There was no G × B interaction effect ($p = 0.1355$). Similarly, genotype ($p<0.0001$), storage bag ($p = 0.0361$) and G × B interaction effect ($p = 0.0486$) had significant effect on release of p-coumaric acid after 8 month of storage. The increase in free phenolic acids during storage might be due to release of bound phenolics that were previously esterified to cell-wall components (Ziegler et al., 2018). Previous studies in rice have reported similar increases in free phenolics during storage and subsequent effects on cooking properties of rice (Tsugita et al., 1983; Ziegler et al., 2018).

The increase of free phenolic acids seems to be associated with the decrease in both PV and FV. After 8 months PV for porridge from OPVI stored in PICS-oxy was higher than PV from grains stored in woven bags. Concomitantly, flour stored in woven bags had significantly higher increase in free ferulic and *p*-coumaric acid suggesting that increase in free phenolics might have contributed to the decrease in PV and FV similarly to previous reports (Li et al., 2018). Similarly for OPVII, woven bag which had the highest free phenolics compared to PICS-oxy had lower PV. To strengthen this postulation flour from woven bags stored grains, which had higher free ferulic acid after 8 months storage of OPVII and white maize genotypes, had significantly lower PV than PICS-oxy. Taken together, these data and those from other studies (Tsugita et al., 1983; Ziegler et al., 2018; Li et al., 2018) support the notion that free phenolic acids may contribute to the modification of flour functionality by affecting starch pasting properties.

Table 3.1. Changes in free ferulic and *p*-coumaric acids (µg/g) during 8 month storage in PICS and woven bags[1]

Genotype	Bag	Free ferulic acid	Free *p*-coumaric acid
OPVI	Initial	31.4±6.7b	0.2±0.0b
	PICS-oxy	48.9±5.7a	0.3±0.1b
	Woven	60.1±7.9a	0.6±0.1a
OPVII	Initial	39.9±0.0b	0.4±0.0a
	PICS-oxy	48.3±11.3b	0.5±0.1a
	Woven	74.9±21.9a	0.5±0.2a
White	Initial	16.2±2.6b	0.8±0.0a
	PICS-noxy	43.4±1.8a	0.6±0.1b
	Woven	35.0±11.9ab	0.8±0.1a

[1]Means with different letters within the column per genotype are significantly different Tukey test $p<0.05$.

3.7.1 Structural changes related to starch during storage in bags

To further explore the potential for structural changes to starch, including potential for phenolic-starch interactions, through storage, we explored changes in starch based on molecular vibration (Li-Chan 1996). Raman spectroscopy was used to study molecular changes in starches. Decreases in viscosities may result from starch whose molecular integrity has been altered during processing procedures such as irradiation (Kizil et al., 2002). These changes are characterized by modification to specific chemical groups (C-H, C-C-C) and linkages such as glycosidic linkages along the starch polymer. These alterations are reflected by changes in Raman intensity and shift of Raman bands (Liu et al., 2015). Therefore, we hypothesized that storage of maize may induce such changes which may also likely contribute to observed changes in pasting profiles.

Starch produces two intense spectral bands at 478 cm^{-1} and 2911cm^{-1} that are used to detect structural changes related to vibrations in the pyranose ring of glucose (C-C-C) and C-H stretching, respectively, (Almeida et al., 2010; Wang et al 2015; Kizil et al., 2002). Storage of maize in PICS bags as well as woven bags altered these intensities suggesting structural changes induced by storage (Figure 3.4). These changes, however, could be induced by, among other factors, starch-phenolic acid complexation (Li et al., 2018). Li *et al* (2018) reported FTIR spectral change resulting from starch-phenolic acids complex using IFTR spectroscopy and

found that complexation of starch with phenolic acid decreased viscosities. Flour from grains with lowest Raman spectra (stored grains) had higher free ferulic and *p*-coumaric acids suggesting a possible relationship between intensity of Raman spectra and free phenolic acid content. Consistent with our results, there was reduction in FTIR spectra at both 995/1022 cm^{-1}/cm^{-1} and 1047/1022 cm^{-1}/cm^{-1} when maize amylopectin and potato starch was complexed with ferulic acid compared with native potato starch and maize amylopectin (Li et al., 2018). Generally, PV was lowest in porridges that had higher free ferulic acids and lower Raman spectral intensity (stored grains) compared to initial grains suggesting a relationship between these three parameters. Raman intensities were dependent on type of storage bag with initial grains producing higher intensities than stored grains (Figure 3.4).

We also observed series of structural changes in the region between1470-850cm^{-1} (Figure 3.4, Table 3.2). This is the region where Raman spectra of carbohydrates present several vibrational features due to coupled vibration involving hydrogen atoms (Almeida et al., 2010). Clearly storage decreased band intensity at 940 cm^{-1} relative to initial grains. This band is assigned to the amylose α-1,4 glycosidic linkage (Almeida et al., 2010; Kizil et al., 2002). Consistent with our result, this band also decreased when native potato starch was complexed with phenolic acids (Li et al., 2018) suggesting a possibility of phenolic acid to modify α-1,4 glycosidic linkage orientation. Another band modified during storage is at 1263 cm^{-1} related to -CH_2OH side chain on pyranose ring C6 position (Table 3.2). All bands in this region showed that storage significantly reduced Raman intensities indicating structural changes. It is not clear from this data whether these structural changes might have contributed to changes in pasting profiles of flour independent of other factors. However, phenolic acid complexation with both maize amylopectin and potato starch reduced PV (Li et al., 2018). Therefore, studies are merited to deduce whether these structural changes affect starch pasting profiles independent of other factors.

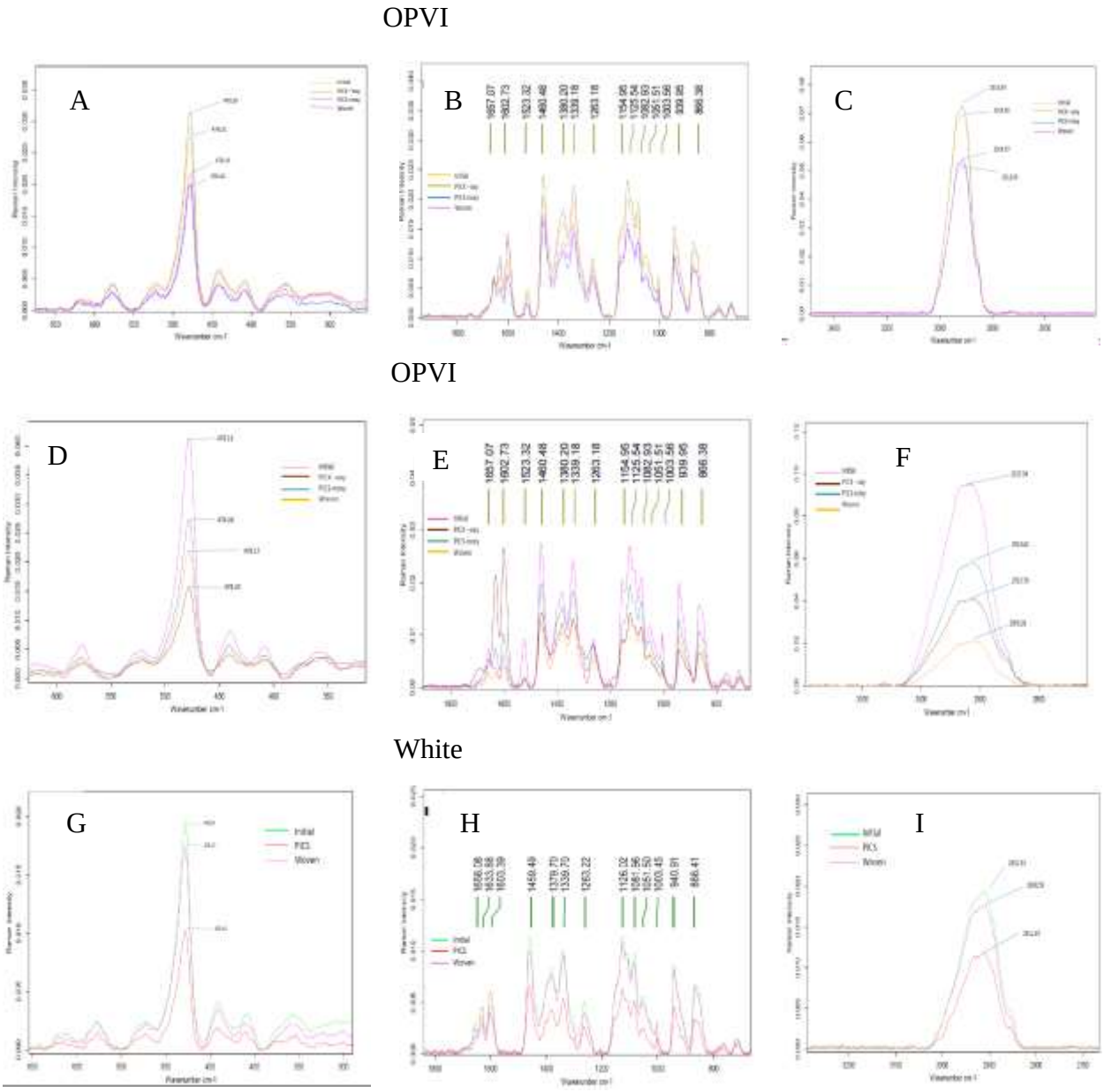

Figure 3.4. Changes in Raman intensities at 478cm^{-1} (A, D,G), between 1470-850cm^{-1} (B,E,H) and at 2911cm^{-1} (C,F,I) for OPVI , OPVII and white maize after 8 months of storage in PICS-oxy, PICS-noxy and woven bags compared to initial grains

Table 3.2: Bands with structural changes in the region 3000-450 cm during storage of maize and their assignment based on literature[2,3,4].

Wave number	Raman Band Assignment	Genotype			Reference
		OPVI	OPVII	White	
2911	C-H stretching	+++	+++	+++	Kizil et al., 2002; Almeida et al., 2010; Liu et al., 2015
1460	CH_2 stretching	++	++	++	Kizil et al., 2002; Almeida et al., 2010
1339	C-O-H bending, CH_2 twisting	++	++	++	Kizil et al., 2002; Almeida et al., 2010
1263	CH_2OH side chain related mode	+	+	+	Kizil et al., 2002; Almeida et al., 2010
1122, 1127	C-O stretching, C-O-H bending	++ (1126)	++(1126)	++(1126)	Kizil et al., 2002; Almeida et al., 2010
1083	C-O-H bending	++	++	++(1085)	Kizil et al., 2002; Almeida et al., 2010
1054	C-O-H, C-O, C-C	++	++ (1051)	++ (1056)	Almeida et al., 2010
1003	unidentified	+	+	+	
940	Skeletal mode vibration of α-1.4 glycosidic linkage, (C-O-C)	++	++	++	Kizil et al., 2002; Almeida et al., 2010
863	C(1)-H, CH_2 deformation	++	++(866)	++(865)	Kizil et al., 2002
478	Skeletal mode of pyranose ring (C-C-C)	+++	+++	+++	Kizil et al., 2002; Almeida et al., 2010; Liu et al., 2015

[2]Band intensities: +++ very strong, ++ strong, + medium, - absent,

[3](parenthesis) indicates exact peak wavelength in this study.

[4]Corresponding wavenumbers for the peaks in the region of 3000-350 cm^{-1} as reported from literature.

Storage of maize results in significant changes related to starch, protein and phenolic acids that affect flour rheology and functionality. These changes are evoked depending on the storage system. Storage of maize in PICS bags has shown to increase FV relative to woven bag.

This effect seems to depend on reduced formation of disulfide linkages and release of free phenolic acids relative to storage in woven bags. The increase in FV by PICS bags has an economic benefit as proportionately less flour would be required to achieve similar viscosities of cooked porridge compared to flour from woven bag stored grains. Storage of maize also results into chemical modification of functional groups on glycosyl residues on starch polymer. Whether these changes affect pasting profiles independent of other factors still remains unknown. Therefore, further studies are merited to understand the contribution of such changes to starch pasting profiles. Here we report that storage of maize in PICS bags does not have any adverse effect on flour functionality but rather a potential economic benefit due to expected proportionately less flour requirement to make porridge with the same viscosity as from grains stored in woven bags.

CHAPTER 4. POST-HARVEST STORAGE ALTERS CAROTENOID BIOACCESSIBILITY FROM BIOFORTIFIED MAIZE

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4.1 Abstract

To better understand if post-harvest storage impacts carotenoid bioaccessibility, two biofortified maize genotypes (OPVI and OPVII) were stored as described in Chapter 3 for 8 months using three different postharvest systems; PICS-oxy (containing oxygen scavengers), PICS-noxy (without oxygen scavengers) and traditional woven polypropylene bags. Carotenoid bioaccessibility was assessed from wet cooked porridges made from 'fresh' and stored grains using an established three stage in-*vitro* digestion model. Relative carotenoid bioaccessibility (% micellarization) was generally higher (2.4-33,7%) in less viscous porridge made from grains stored in woven bags compared to porridge from initial (2.3-22.5%) or PICS stored grains (2.0-27.4%) suggesting an impact of ageing and storage type. Further higher viscosity porridges might partly explain lower relative bioaccessibility in fresh grain or PICS stored grain porridges. Absolute carotenoid bioaccessibility from experimental porridge was dependent on carotenoid species and storage system from which flour was derived. Calculation of absolute bioaccessibility (µg of bioaccessible pVA per g of flour) suggests that initial grains would provide more bioaccessible carotenoids (0.32-1.29 ug/g) compared to stored grains (0.17-0.49, 0.15-0.45, 0.18-0.63 ug/g in PICS-oxy, PICS-noxy and woven bags respectively) and that storage losses remain the primary factor impacting total available carotenoids. However, when considering the enhancement of relative bioaccessibility in stored grains, presumably through aging effects and digestibility, absolute levels of bioaccessible carotenoids from biofortified maize products did not differ significantly between storage systems. Therefore, post-harvest storage but not storage system appears to be the main factor negatively impacting the overall levels of carotenoids made available for absorption.

4.2 Introduction

Beyond efforts to enhance provitamin A carotenoid content and post-harvest stability in biofortified maize, consideration of ultimate bioavailability of these micronutrients is critical to delivery of the nutritional benefit. Carotenoid bioavailability is defined as the portion of carotenoids absorbed from food and that made available for utilization, metabolism and storage by the human body (Parker et al., 1999; Goltz et al., 2012). Carotenoid absorption occurs through several sequential stages that include (1) release from food matrix by normal digestion, (2) incorporation into bile salt mixed micelles requiring co-consumption of fat, (3) passive and facilitated uptake of micelles by intestinal epithelia, (4) package of carotenoids into chylomicron and (5) final secretion in lymphatic system and transfer to the blood stream (Ball, 1998; Goltz et al., 2012).

Bioavailability of carotenoids is influenced by food matrix/dietary factors including the type and amount of co-consumed lipid and the type and extent of food processing (Van het Hof et al., 1999; Goltz et al., 2012). In this context, bioaccessibility is defined as the proportion of carotenoids that are released through normal digestion and transferred into bile salt mixed micelles and made available for absorption (Goltz et al., 2012). Carotenoid bioaccessibility is often used as a surrogate of bioavailability as it is (1) easily measured using in vitro model systems and (2) has been shown to be highly predictive of carotenoid bioavailability in humans (Reboul et al., 2006). In this regards, screening of carotenoid bioaccessibility allows for assessment of food matrix factors that may impact ultimate absorption in humans, and thus makes it highly applicable to screening germplasm collection, or in this case, impacts of factors such as post-harvest storage.

Overall, carotenoid bioaccessibility is highly variable between different food matrices with carotenoids from fruit and maize reportedly having higher bioaccessibility than those from dark-green leafy vegetables and carrots (de Pee et al., 1998; Castenmiller and West 1998; Hedren et al., 2002). Carotenoid bioaccessibility (micellarization efficiency) from maize are reportedly higher (Thakhar and Failla, 2008; Kean et al., 2008) than from vegetables (Castenmiller and West 1998; de Pee et al., 1998) though comparison between studies are difficult because of different methodological aspects and general lack of reference standards or controls. However, these results are consistent with reports on carotenoid bioavailability from maize (16.7%), orange

fleshed sweet potato (0.6-73%), carrots (19-34%) and broccoli (22-24%) being higher than those from green leafy vegetables (3-6%), (Thakkar and Failla, 2008; Bechoff et al., 2011; Failla et al., 2009; Mills et al., 2009; Brown et al., 1989; Micozzi et al., 1992; De Pee et al., 1995; Torronen et al., 1996; Castenmiller and West, 1999; van het Hof et al., 1999). Bioaccessibility of maize carotenoids varies depending on type of processed maize product. For example, micellarization efficiency of xanthophylls from yellow cornmeal extruded puff was higher (63-69%) than from yellow cornmeal porridge (48%) (Kean et al., 2008) suggesting process inducted changes in digestibility or other factors may aid in release of carotenoids in the GI tract. In the same study micellarization efficiency of xanthophylls from whole yellow cornmeal was highest in bread (85%) and lower in extruded puff (46%) and porridge (47%). β-carotene had the lowest micellerization efficiency in puff and bread (11-23%) but higher in porridge (40-63%) (Kean et al., 2008). All in all this suggest that food form as well as material combine to impact the ultimate release and bioaccessibility of maize carotenoids.

Beyond processing, storage of maize may be another factor to consider. Post-harvest storage conditions can be variable in regions where provitamin A biofortified maize are targeted as a strategy to alleviate vitamin A deficiency. Long-term storage of biofortified maize results into loss of carotenoids predominantly due to oxidative degradation (Chapter 2 and Ortiz et al., 2016). What is not well understood is the effect of storage on bioaccessibility of various carotenoids from common African products such as porridges. To date, no study has specifically looked at the effect of storage of biofortified maize on carotenoid bioaccessibility. Our work has shown that storage of biofortified maize results into significant physico-chemical changes that affect derived flour functionality (Chapter 3). These changes are induced and enhanced by storage environment that include temperature and humidity (Setiawan et al., 2010). The major changes occurring in cereals during storage are those related to starch and protein. These changes are not only structural but also nutritional. Starch tends to becomes more crystalline (Awazuhara et al., 2000; Setiawan et al., 2010) while proteins tend to become more polymerized through disulfide linkages (Griffin and Hamaker, 1990). These changes can affect digestibility of starch and protein (Rehman, 2006) and therefore may be assumed to also affect bioaccessibility of other nutrient components that are found associated with these macronutrients i.e carotenoids. Here, we hypothesize that physico-chemical changes occurring during storage of grains as characterized in Chapter 2, would also lead to a reduction in carotenoid bioaccessibility relative

to initial non-stored samples. Therefore, this study aims to assess the effect of storage of biofortified orange maize for 8 months on carotenoid bioaccessibility.

4.3 Methodology

4.3.1 Storage of maize

Detailed description of maize genotypes OPVI and OPVII as well as storage conditions of maize have been previously described (Nkhata et al., 2019). Briefly, two biofortified orange maize genotypes were harvested and dried to ~8% moisture and then packed in PICS bags with oxygen scavengers enclosed (PICS-oxy), PICS bags without oxygen scavengers (PICS-noxy) and polypropylene woven bag (Woven) for 8 months. All bags were stored under same conditions; 29 ^{o}C and 30% relative humidity (RH). After 8 months, representative samples were taken from each bag and stored at -80^{o}C until further processing. Milling and analyses were initiated within one week of sampling.

4.3.2 Preparation of experimental porridge

Porridge preparation followed the general method described by Lipkie et al., 2013. Briefly, 10 g maize flour was slurried in 20 mL cold distilled water. This slurry was added to 20 mL of boiled water and the mixture was cooked for 5 minutes at 100 ^{o}C with occasional stirring. The porridge was allowed to cool under room temperature for 10 minutes thereafter was taken for immediate carotenoid extraction. This formulation gave porridge dry matter content of ~25.2%. Approximately 2 g of porridge was used for moisture analysis.

4.3.3 In vitro digestion for determination of carotenoid bioaccessibility

Carotenoid bioaccessibility was assessed using the *in vitro* digestion model as described by Lipkie et al. (2013). Briefly, ten grams (10 g) of porridge containing ~10% canola oil was combined with 6 mL of oral phase base solution with α-amylase and incubated at 37 ^{o}C for 10 minutes under nitrogen in a shaking incubator (Oral Phase). For the Gastric Phase, the pH of the oral digesta was adjusted to 4.0 using 1.0 N HCl and 2 ml of 10 mg/ml pepsin was added. The pH was adjusted to 2.5 using 1.0 N HCl and the mixture was incubated for 1 hour at 37 ^{o}C under nitrogen. Following gastric digestion the intestinal phase was initiated by adjusting the pH to 4.0 with 1 N $NaHCO_3$ and then adding 2 ml of 20 mg/mL pancreatin (0.8 g/L), 10 mg/mL lipase (0.4

g/L) and 3 mL of bile extract solution (1.8 g/L). Final pH was adjusted to 6.5 with 1.0 N $NaHCO_3$. The mixture was incubated at 37 ^oC in shaking incubator for 2 hours**.** After incubation aliquot for each sample digesta (DG) was transferred to polycarbone tube for high speed centrifugation (10,000×g for 1 hour). Finally, aqueous fraction (AQ) was syringe filtered through 0.22 mm filters and stored at -80 ^oC until carotenoid extraction the following day.

4.3.4 Carotenoid extraction and LC-DAD

Maize carotenoids were extracted as previously reported (Ortiz et al., 2016). Briefly, thawed aliquot (10 mL) of DG and AQ was extracted 2 times with 1 mL acetone and 3 mL petroleum ether containing 0.1% BHT then dried under nitrogen. AQ was resolubilized in 100 µL of a mixture of ethyl acetate and methanol in a 1:1 ratio while DG was resolubilized in 500 µL of a mixture of ethyl acetate and methanol in a 1:1 ratio for immediate analysis. The injection volume was 10 µL. Carotenoid separation was carried out on Acquity H Class UPLC (Milford MA, USA) equipped with a Acquity Photodiode Array eLambda detector.

4.3.5 Data analysis

Carotenoid content for raw material, digesta (DG) and aqueous (AQ) represent mean and standard deviation from a minimum of three replicates. Carotenoid relative bioaccessibility was calculated using formula (1) while carotenoid absolute bioaccessibility was calculated using formula (2) below;

$$\text{Relative bioaccessibility (\%)} = \frac{\text{Carotenoid content of AQ}}{\text{Carotenoid content of DG}} * 100. \quad (1)$$

$$\text{Absolute bioaccessibility } (\mu g/g) = \frac{\text{Carotenoid content in AQ}}{\text{Carotenoid content in DG}} * 200g \text{ porridge serving.} \quad (2)$$

Data were analyzed by running ANOVA on SAS 94 version (SAS Institute Inc, NC) to generate means±standard deviations of carotenoid content in porridges made from maize stored in three different bag systems (PICS-oxy, PICS-noxy and woven bags) and then compared with carotenoid content in porridge made from initial grains. Significant differences were determined using Tukey post hoc test when $p < 0.05$.

4.4 Results and discussion

The carotenoid content of maize grains used for preparation of experimental porridges are presented in table 4.1. Similar to our previous report (Nkhata et al., 2019), PICS-oxy stored grains retained more carotenoids ($p<0.05$) than woven bag stored grains for OPVI. However, there were no significant differences in carotenoid contents between PICS-oxy stored grains and woven bag stored grains for OPVII. As expected, 'initial' grains had significantly higher ($p<0.05$) carotenoid content than 'stored' grains for both genotypes.

Table 4.1.Carotenoid content (μg/g dry weight) of OPVI and OPVII grains used for preparation of test porridge[1,2,3]

			Month 8	
Carotenoids	**Genotype**	**Initial**	**PICS-Oxy**	**Woven**
Lutein	OPVI	3.30 ±0.10a	2.57 ± 0.23b	1.47 ± 0.15c
	OPVII	4.83 ±0.31a	3.00 ± 0.26b	2.57 ± 0.38b
Zeaxanthin	OPVI	15.13 ± 0.58a	11.73 ± 1.37b	7.17 ± 0.55c
	OPVII	8.43 ±0.37a	4.80 ± 0.53b	4.47 ± 0.74b
β-Cryptoxanthin	OPVI	1.60 ± 0.00a	1.30 ± 0.17b	0.97 ± 0.06c
	OPVII	1.43 ± 0.06a	0.97 ± 0.06b	0.97 ± 0.12b
trans-β-carotene	OPVI	2.43 ±0.06a	1.83 ± 0.23b	1.53 ± 0.03b
	OPVII	2.73 ± 0.06a	1.60 ± 0.10b	1.53 ± 0.06b
Total pVAC	OPVI	4.03 ± 0.04a	3.13 ± 0.21b	2.50 ± 0.04c
	OPVII	4.16 ± 0.05a	2.57 ± 0.07b	2.50 ± 0.07b
Total carotenoids	OPVI	22.5 ±0.12a	17.4 ± 0.80b	11.14 ± 0.36c
	OPVII	17.42 ± 0.22a	10.37 ± 0.31b	9.54 ± 0.25b

[1]Carotenoid content (mean ± SD) at each testing interval in different bags $n = 3$. [a-b]
[2]Means with different letters within a row are significantly different Tukey test ($p<0.05$).
[3]*cis-β*-carotene = 15-*cis*- *β*-carotene + 13-*cis-β*-carotene + 9-*cis-β*-carotene. pVAC = *β*-cryptoxanthin + cis-*β*-carotene + trans-*β*-carotene.

Porridges were made from stored grain to determine not only the effect of storage on bioaccessibility of carotenoids but also comparative carotenoid bioaccessibility of different

storage system. Lutein relative bioaccessibility (% micellarization) was generally higher than that of zeaxanthin in all storage bag systems and for both genotypes (Figure 4.1). Lutein and β-carotene relative bioaccessibility was higher in woven bags than other storage bag systems for both genotypes. However, unexpectedly, the relative bioaccessibility of both lutein and zeaxanthin were found to be low (2.0-16.9%), relative to previous report from maize porridge (27.9-85.0%) (Kean et al., 2008; Dube et al., 2018; Thakkar and Failla, 2008). The reason for this low level of micellarization for xanthophylls is not clear as micellarization for β-carotene remain consistent if not higher with levels previously reported for yellow maize (Thakkar and Failla, 2008) and transgenic sorghum (Lipkie et al., 2013). Considering these are biofortified grains, it is possible that these lower ranges for lutein bioaccessibility may be a result of differences in carotenoid profiles of these grains. This remains to be further explored.

In all storage systems, β-carotene bioaccessibility was highest (12.9-33.7%) and that of β-cryptoxanthin appeared to be less affected by storage system. Comparatively, relative carotenoid bioaccessibility from initial grains was higher in OPVI than OPVII (Figure 4.1). As stated previously, all relative carotenoid bioaccessibilities reported in this study are generally lower than those reported previously (Thakkar and Failla, 2008; Dube et al., 2017; Kean et al., 2008). However, in all these studies the lutein+zeaxanthin to β-carotene or β-cryptoxanthin ratios in the genotypes were far lower than the ratio in the genotypes used in this study which have more than ~9 folds higher content of lutein + zeaxanthin than β-carotene or β-cryptoxanthin. Whether the high content of lutein and zeaxanthin is responsible for lower relative bioaccessibility in these genotypes still remains unknown as data supporting the notion that high lutein and zeaxanthin reduce bioaccessibility of other carotenoids remains inconsistent (Thakkar and Failla, 2008; Dube et al., 2017; Davis et al., 2008; Diaz-Gomez et al., 2017).

Absolute carotenoid bioaccessibility (µg/200g serving) from experimental porridges was generally higher for lutein, zeaxanthin and β-carotene than for β-cryptoxanthin (Table 4.2) though the relative bioaccessibility for zeaxanthin was the lowest (Figure 4.1). Porridges from OPVI initial grains had significantly higher absolute carotenoid bioaccessibility compared with porridge from OPVII initial grains (Table 4.2) suggesting the potential for some genotypic differences. This genotypic effect was less evident in grains stored in PICS-oxy, PICS-noxy and woven bags. While storage system had significant effect ($p<0.05$) on absolute carotenoid

bioavailability from porridges, it did not have any clear or specific trend on grains stored in three different bag systems based on either storage system or genotype (Table 4.2).

The higher lutein and β-carotene relative bioaccessibility from woven stored grains was unexpected but may be related to the reduction in overall carotenoid content over time and therefore suggestive of a potential for a concentration dependent effect. With the carotenoid content in the stored grains being lower it is possible for a higher percentage that may fall within range of micellarization levels. However, this has to be further explored to ascertain the likely cause of this observation. Moreover, the effect of serving viscosity (final viscosity) of the porridge should also be examined. Porridges made from grain stored in woven bags had generally lower final viscosities than PICS bag stored grains (Table 4.3). Low viscosity could mean that the porridge was more easily digested and easily accessible to enzymes compared to high viscous porridge from PICS bags. Bioaccessibility and bioavailability of carotenoids are highly affected by the viscosity of the food matrix with highly viscous food reducing carotenoid bioaccessibility significantly (Gallaher et al., 1993; Desmarchelier and Borel, 2017). The model utilized in this study is a static model and the effects of viscosity differences in the food may impact ultimate digestibility. While conditions used were optimized for fresh porridges, differences in real digestibility between these could explain, in part, the differences in bioaccessibility.

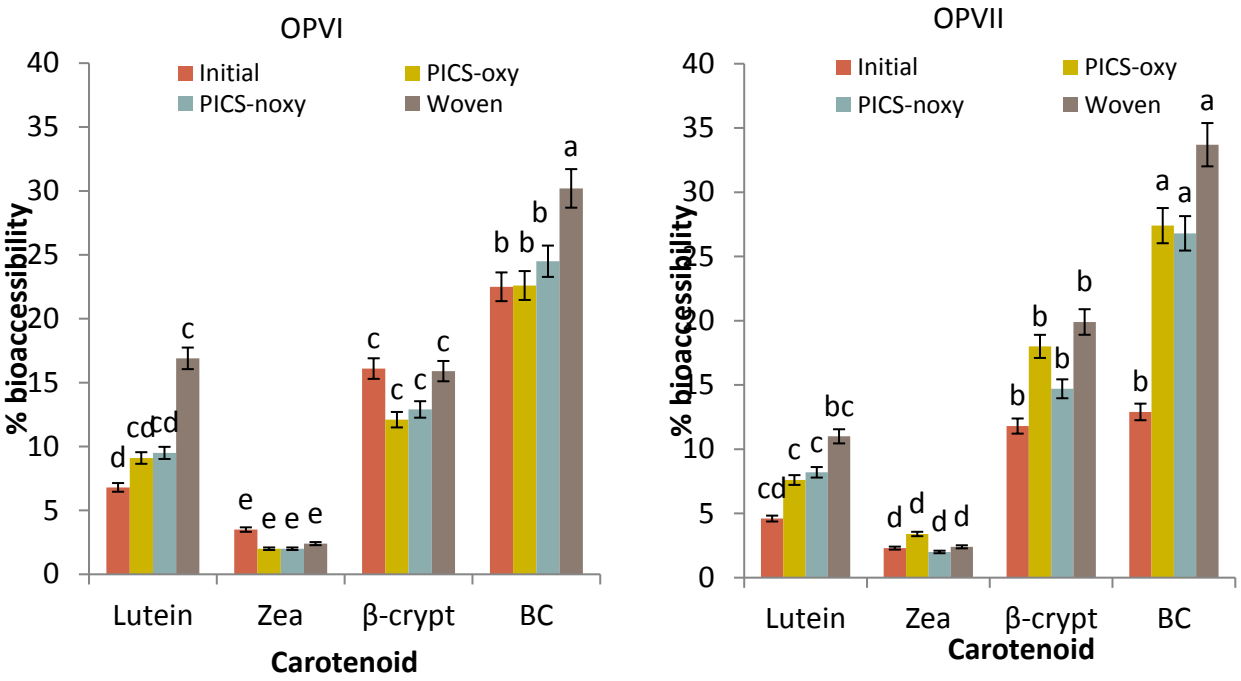

Figure 4.1. Relative carotenoid bioaccessibility from experimental porridge prepared from grains stored for 8 month in different storage bags. Initial: porridge from non-stored grains; PICS-oxy: porridge from grains stored in PICS-oxy bags; PICS-noxy: porriddge from grains stored in PICS-noxy bags; woven: porridge from grains stored in woven bags. Each bar is an average of $n = 3$ replicates. For each genotype, bars with different letters are significantly different according to the Tukey's test ($p<0.05$).

Table 4.2. Absolute carotenoid bioaccessibility in 200 g experimental porridge (µg/200 g serving porridge)[4,5,6]

Carotenoid	Genotype	Initial	PICS-oxy	PICS-noxy	Woven	p-value
Lutein	OPVI	40.0 ± 2.0*	28.0 ± 4.7	26.0 ± 4.4	34.2 ± 4.2	*0.2634*
	OPVII	21.6 ± 3.0b*	25.4 ± 2.4ab	28.3 ± 3.7ab	30.0 ± 2.7a	*0.0408*
Zea	OPVI	35.1 ± 2.8a*	25.0 ± 3.5ab*	18.6 ± 3.0b	22.5 ± 6.5ab	*0.0302*
	OPVII	18.9 ± 3.6 *	17.7 ± 3.7 *	16.9 ± 4.0	17.9 ± 3.9	*0.9475*

Table 4.2 continued

Carotenoid	Genotype	Initial	PICS-oxy	PICS-noxy	Woven	p-value
β-crypt	OPVI	13.5 ± 0.1a*	13.6 ± 0.6a	11.1± 0.5b	13.0 ± 0.4a	*0.0037*
	OPVII	11.6 ± 0.2b *	12.6 ± 0.3ab	12.5 ± 0.4ab	13.0 ± 0.4a	*0.0093*
all-trans-BC	OPVI	30.4 ± 0.6a*	31.0 ± 0.7a	25.8 ± 0.6b*	30.7± 0.1a	*0.0004*
	OPVII	26.5 ± 0.9b*	29.0 ± 0.5a	29.2 ± 0.8a*	30.8 ± 0.2a	*0.0007*

[4]Means with different letters within a row are significantly different Tukey's test $p<0.05$.
[5]Abbreviations: Zea; Zeaxanthin, β-crypt; β-cryptoxanthin, *all-trans*-BC; *all trans*-β-carotene.
[6]Presence of * indicates that absolute carotenoids bioaccessibility are significantly different between OPVI and OPVII, Tukey's test ($p<0.05$).

Table 4.3. Final viscosities (serving viscosity) of porridge made from initial grains and from grains stored in PICS-oxy, PICS-noxy and woven bags stored for 8 months.

Genotype	Initial	PICS-oxy	PICS-noxy	Woven
OPVI	866.7±38.4bc	1060.0±55.2a	1007.3±86.4ab	826.7±24.0c
OPVII	1069.3±56.6ab	1186.3±63.7a	958.5±7.8b	386.0±33.9c

Means with different letters within a row are significantly different Tukey's test $p<0.05$.

Storage of biofortified maize decreased the overall levels of carotenoids made available for absorption. Though relative carotenoid bioaccessibility seems to be higher in grains stored in woven bags than initial grains and those stored in PICS bags, conversion of these percentages into μg/g of the final content in grains could provide a clearer picture of the quantity potentially available for absorption. In fact, storage but not storage system seems to have significant effect on extrapolated absolute carotenoid bioaccessibility. The higher content of carotenoids in the initial grains compared to stored grains may overcome the increased relative bioaccessibility effect in stored grains. The lower carotenoid content following storage in woven bags relative to PICS bags counteract the potential benefit of increased relative carotenoid bioaccessibility as a result of storage, resulting in insignificant net gain in absolute carotenoid bioaccessibility. This study support the notion that relative bioaccessibility of carotenoids is inversely proportional to

their content in food (Stahl et al., 2002; Schmaelzle et al., 2014). During storage carotenoids were lost with grains stored in woven bag having the lowest contents after storage period. Therefore, this may partly explain higher relative bioaccessibility in porridge made from grains stored in woven bags compared to initial un-aged grains. However, it is doubtful that the carotenoid content (doses) in these grains could elicit such dose dependent effect because absorption of carotenoids are linear up to doses of 20-30 mg (Stahl et al., 2002) which is far much greater than the dose in the maize used in this study. Therefore, further studies are required to ascertain the cause of this observation.

4.5 Conclusion

Storage of high carotenoid biofortified orange maize results in low carotenoid content due to degradation during storage. This affects both relative and absolute carotenoid bioaccessibility from porridges made from stored grains. Though the relative carotenoid bioaccessibility of some carotenoids increased the absolute carotenoid bioaccessibility decreased after storage resulting in low carotenoids available for absorption. The higher absolute carotenoid bioaccessibility in porridges made from initial grains seemed to be related to higher carotenoid content compared to PICS and woven bags stored grains. Storage had more effect on absolute carotenoid bioavailability compared to storage system. The increase in relative carotenoid bioaccessibility from porridge made with stored grains did not overcome effect of carotenoid loss during storage thereby impacting the absolute amounts of carotenoids that are in fact bioaccessibile. Therefore, storage and to a lesser extent storage type, resulted in a decrease of carotenoids that could be made available for absorption.

CHAPTER 5. CONCLUSION AND FUTURE DIRECTION

The overall goal of this research was to understand the chemical and physical changes in biofortified maize through postharvest storage and specifically, to assess the effectiveness of the Purdue Improved Crop Storage (PICS) system in maintaining food and nutritional quality of biofortified maize. PICS bags was developed for and promoted in developing countries for post-harvest storage of grains and other crops to alleviate insect and other pest damage. Specifically, PICS works through a multilayer consisting of interior two high density polyethylene (HDPE) and an outer layer made from polypropylene sack that allows for a bio-generated modified atmosphere inside the bags derived directly from respiring pests and grains. As diffusion of gases into and from the bag is limited, consumption of oxygen results in the eventual asphyxiation of pest. Building on the usefulness of this system, we proposed that such a bio-generated modified atmosphere (low oxygen partial pressure) created in PICS bags could be a method to reduce oxidative losses in provitamin A and total carotenoid content of biofortified maize during post-harvest storage. Post-harvest stability of the provitamin A in maize, as well as other biofortified crops, has remained a long term challenge in need of cost effective solutions in order to fully deliver on the promise of vitamin A biofortification.

With this in mind, we designed experiments to assess two biofortified orange maize genotypes (OPVI and OPVII) using three different storage systems over 8 months at 29 ^{o}C and 30 % rh. Specifically, we carried out a comparative assessment of PICS-oxy (with oxygen scavengers), PICS-noxy (without oxygen scavengers) and traditional polypropylene woven bags. Maize grained stored in these bag systems were assessed for nutrient stability (carotenoids) as well as functional properties known to be impacted by ageing/oxidative conditions. Finally, we assessed the potential for alteration of carotenoid bioaccessibility through postharvest storage.

Monitoring changes in oxygen levels within these systems demonstrated that the presence of an oxygen scavenger (PICS-oxy) predictably resulted in decreasing oxygen content during first 15 days compared to maize stored without an active scavenger (PICS-noxy). Somewhat surprisingly, oxygen levels in PICS-noxy remained similar to environmental level (~21%) suggesting that grains alone, without an active insect infestation, were not effective in reducing

oxygen levels. This is most likely due to the low respiration rate of grains and, as stated, these grains were free from storage pests at the point of packaging in storage bags. After 4 months oxygen level in PICS-oxy did increase and ultimately reached levels that were similar to other storage systems tested suggesting that PICS-bags are not perfectly hermetic. Though temperature was similar in all bags and storage room relative humidity (RH) changed within 8 month storage, PICS bags (both PICS-oxy and PICS-noxy) maintained RH throughout storage period. RH in woven bag was variable depending on the atmospheric RH and increased to ~60% by 8^{th} month of storage.

Following this initial measurement of RH and oxygen changes, we assessed and compared the utility of PICS to traditional woven bags for their ability to slow post-harvest carotenoid degradation. After four months of storage, grains stored in PICS-oxy maintained a significantly ($p<0.05$) higher carotenoid content that PICS-noxy and woven bags suggesting that sequestering oxygen inside the bags slowed down carotenoid loss. Carotenoid retention (%) was higher for OPVI than OPVII suggesting the potential genotypic differences. However, after eight months, carotenoid retention did not differ between storage bags. Therefore, it appears that PICS-bags effectiveness in reducing post-harvest degradation of carotenoids is enhanced when oxygen is removed from the bag during closing, however, this benefit is lost as oxygen diffuses back into the bag. Grains with higher physiological activities (respiration) could perhaps reduce oxygen and therefore could provide a practical way of reducing oxygen inside the bag that could increase carotenoid stability. Similarly, some level of infestation, may serve a benefit of consuming oxygen in the early stages of storage. In any case, our experiment did not deal with significant levels of infestation and that question remains outstanding. Still, considering the findings of four month storage, PICS-bags do appear to provide an economical option for storing high carotenoid biofortified maize with some benefit for carotenoid retention in the short term. However, the approach of using physiologically active grains or other strategies to modify oxygen content in bags should be further investigated.

At the same time we investigated the effect of post-harvest storage on rheological properties and aspects of maize flour functionality. Biofortified maize grains stored in PICS-oxy, PICS-noxy and woven bags were ground into flour and Rapid Visco Analyzer was used to generate pasting profiles which represent the cooking performance of flour and subsequent

product texture and physical quality. The pasting profiles for the two biofortified maize differed in several aspects (e.g. peak viscosity, final viscosity) compared to ordinary white maize (more similar to traditional African white maize) suggesting that biofortified maize may not provide same porridge viscosity as white maize when same amount of flour is used. This also indicates that biofortified maize flour may require proportionally more flour to achieve similar viscosity compared to flour from white maize. While viscosities for biofortified maize genotypes after 8 months did not differ with initial viscosities the viscosities for white maize genotypes significantly ($p<0.05$) decreased after eight months suggesting storage had more effect on ordinary white maize than biofortified maize genotypes. However, regardless of genotype, woven bag stored grains produced lowest viscosities than PICS bag stored grains.

To better understand the chemistry involved in these effects, disruption of disulfide linkages using treatments of 10 µM Dithiothreitol (DTT) resulted in an increased Peak Viscosity (PV) and Break Down (BD) but decreased Final viscosity (FV) and Set back (SB). Increase in PV after DTT treatment suggests that DTT disrupted disulfide linkage that restricted starch granule swelling. Increase in BD is indicative that DTT made porridge (paste) more susceptible to disintegration and may suggest roles that disulfide linkages might play in providing stability to the paste. This is also supported by decreased FV and SB in DTT treated samples which probably suggests that disulfide linkage disruption either interferes with starch retrogradation or results in loss of 'stabilizing effect offered by protein' resulting in softer paste. DTT treatment did not level PV in initial samples and samples stored for 4 months suggesting that other factors might have contributed to differences in PV from samples stored in different bag types. Indeed phenolic acid content increased during storage and phenolic acids are known to reduce starch viscosities (Li et al., 2018). At the same time, woven bags had highest free phenolic acid increase, produced lowest viscosities further suggesting a link between free phenolic acid content and pasting profiles. It is also possible that starch-phenolic acid complexation might be responsible for the starch structural changes shown by Raman Spectroscopy. Results from this study shows that biofortified maize genotypes produce porridges with lower viscosities than ordinary white maize. Storage does appear to alter flour pasting profiles but is dependent on the type of storage system. Storage in PICS bags does not appear to adversely affect flour functionality and has some benefit in that it results in flour with higher FV compared to storage in woven bags. Whether the increase in viscosity in PICS bags stored grains could translate into significant

economic or further enhance the goals of food security at household level needs to be further investigated.

Finally we sought to explore the potential effects of storage on bioaccessibility of carotenoids from biofortified maize as this is critical stop in achieving the ultimate aim of maize biofortification: alleviation of vitamin A deficiency in target populations. Experimental porridge formulated to mimic thin porridges commonly consumed in Africa were subjected to a three stage i*n vitro* digestion model designed to mimic normal human digestion and model the extent to which carotenoids are extracted from the food and transferred to the micellar aqueous fraction in the human intestine. While relative carotenoid bioaccessibility (% micellarization) was higher in grains stored in woven bags compared to initial grains and those stored in PICS-oxy and PICS-noxy bags, absolute carotenoid bioaccessibility from porridge generally remained higher in fresh grain samples by virtue of the higher carotenoid content. Further, the extrapolated carotenoid bioaccessibility based on content in grains revealed that initial grains could provide more absolute bioaccessible carotenoid than stored grains regardless of storage system. Higher relative carotenoid bioaccessibility in grains stored in woven bags maybe related to lower content in grains as lower carotenoid content may have higher micellar incorporation efficiency than higher contents (dose effect). Similarly, high absolute carotenoid bioaccessibility in initial grains is related to higher carotenoid content relative to stored grains. Therefore storage results in an anticipated decrease in absolute carotenoid bioaccessibility suggesting that stored grains may indeed have lower nutritional value than fresh grains. The increase in relative carotenoid bioaccessibility is counteracted by the low carotenoid content due to degradation in stored grains compared to initial resulting in insignificant net effect on bioaccessibility.

While increasing carotenoid content in biofortified maize genotypes is important, slowing down degradation of carotenoids during storage will improve the efficacy of biofortified maize in delivery of provitamin A for target population. To achieve this, the population must accept and consume the biofortified maize and their derived products. This calls for development of products that will be accepted, therefore, understanding the performance of biofortified maize flour in food system is important. While the literature suggests biofortified maize is acceptable, it is important to determine what drives consumer liking of biofortified maize and optimize those attributes in development of new products. Consuming highly nutritious biofortified maize and

its derived products with bioaccessible provitamin A carotenoids will contribute to alleviation of VAD in developing countries.

Beyond this work, future effort should focus on identifying most economical mechanisms of lowering oxygen level inside PICS bags. Our suggestion that more physiologically active grains and use of insect infestation instead of chemical oxygen scavengers for initial drop of oxygen should be explored. Since PICS bags maintain the relative humidity the optimum humidity for PICS bag storage should also be identified on a wide range of genotypes and under different storage conditions. While color is the noticeable aspect that differentiates white from biofortified orange maize there is limited information regarding what drive consumer liking or disliking of orange maize. Our study has shown that some biofortified maize genotypes may provide a less viscous porridge than some white maize genotype. Whether these aspects are relevant to increased adoption of biofortified maize still remains unknown. Therefore, a qualitative descriptive analysis of various products derived from biofortified orange maize is needed.

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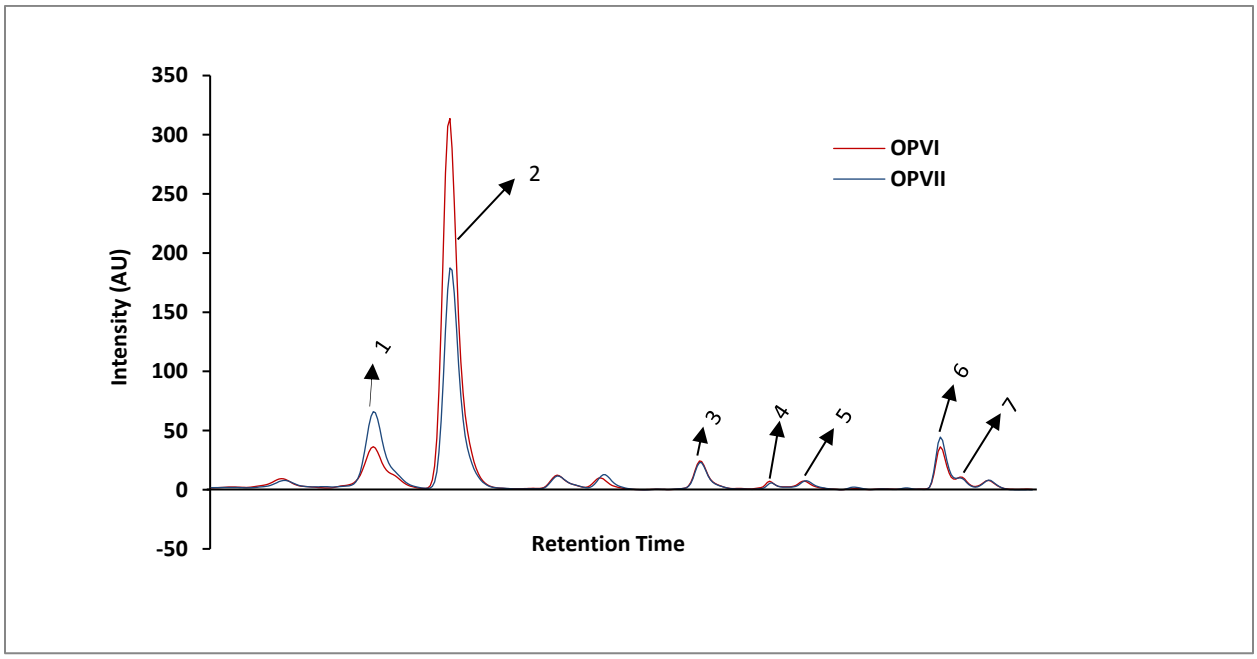
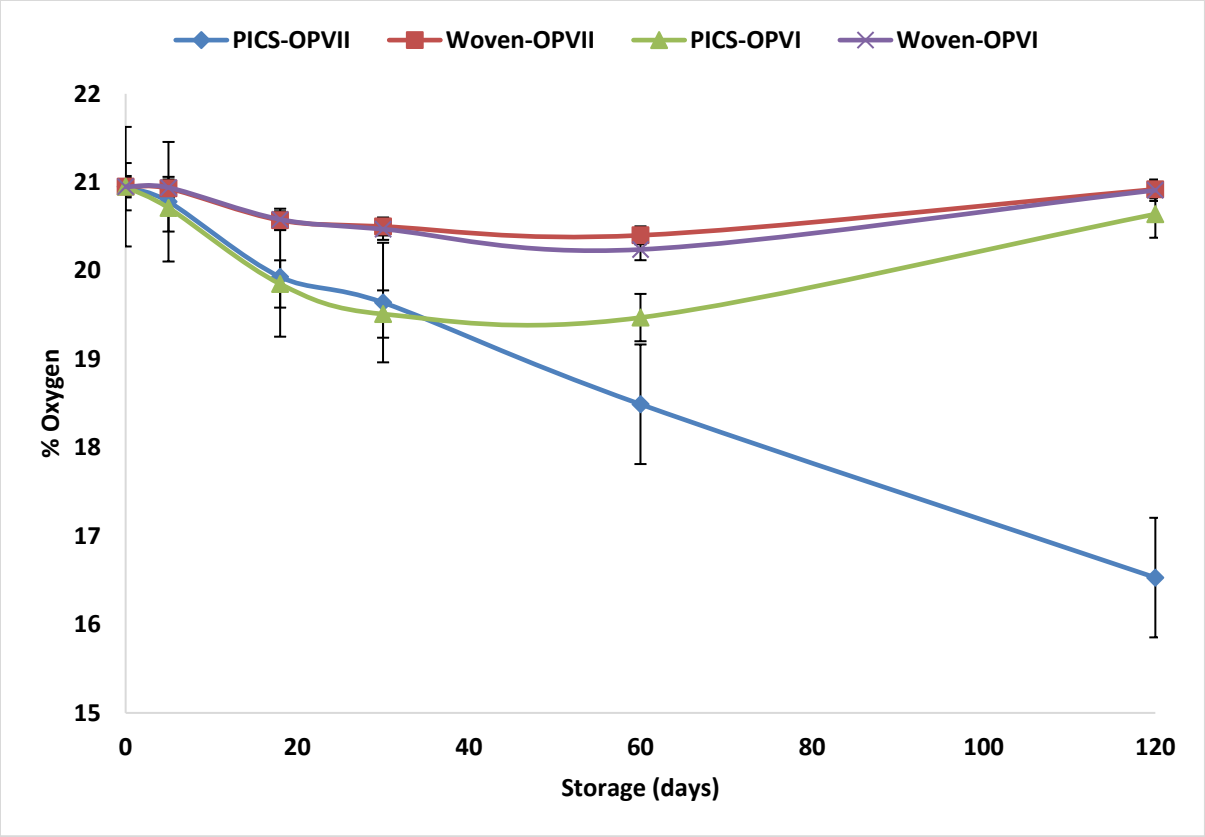
APPENDIX A. SUPPPLEMENTARY FIGURES AND TABLES



Supplamental Figure 1. Chromatograms showing relative initial carotenoid concentration for OPVI and OPVII genotypes. 1. Lutein, 2. Zeaxanthin, 3. *β*-cryptoxanthin, 4. 15-*cis*-*β*-carotene, 5. 13-*cis*-*β*-carotene, 6. All *trans*-*β*-carotene, 7. 9-*cis*-*β*-carotene.



Supplemental Figure 2. Reduction in oxygen in PICS and Woven bags during 4 months storage for OPVI and OPVII.

Supplemental Figure 3. Differences in moth infestation of maize grains stored for 8 months. 1, grains stored in PICS bags. 2, selected grains from woven bags showing germs attacked by moths (pointed by arrows). 3, Grains stored in woven bags (sticker on data logger eaten by moths).

Supplementary Table 1. Pasting profiles of flours treated with or without DTT after storage for 4 months

Genotype OPVI

Treatment	PV	T	BD	FV	ST	PT	PTemp
Initial no DTT	430.3±19.0d	399.3±12.3c	31.0±19.2c	866.7±38.4c	467.3±26.7cb	4.78±0.3bc	77.8±0.6a
Initial DTT	559.0 ± 0.0c	140.0±8.9e	419.0 ±10.2b	396.5± 5.1e	257.0±8.8e	4.0±0.0c	75.0±0.0b
PICS-oxy no DTT	602.3± 16.9c	570.7±10.1a	31.7±17.2c	1151.7± 34.4a	581.0±24.3a	6.3±0.5a	74.0±1.2b
PICS-oxy DTT	879.7 ±30.1a	307.3±29.5d	572.0±8.7a	684.3±44.8d	376.7±23.0cd	4.1±0.0c	73.7±0.4b
PICS-noxy no DTT	557.7 ±34.2c	539.3±34.1ab	18.3±7.0c	1116.3± 42.5ab	577.0±23.1a	5.9±0.7ab	73.2±0.4b
PICS-noxy DTT	811.0± 55.ab	223.3±42.1de	587.7±21.0a	553.7± 86.9de	330.3±44.8de	4.0±0.0c	73.4±0.0b
Woven no DTT	497.3 ±16.3cd	480.7±19.2bc	17.7±2.3c	955.0 ±39.0bc	475.3±22.2b	6.1±0.4a	77.5±0.0a
Woven DTT	756.0± 56.1b	197.7±32.1e	558.3±24.1a	516.0± 70.5de	318.3±38.7de	4.0±0.0c	73.6±0.4b

Genotype OPVII

Treatment	PV	T	BD	FV	SB	PT	P Temp
Initial no DTT	594.0±37.7de	502.7±36.5ab	91.3±8.1c	1069.3±56.6ab	566.7±23.4ab	4.7±0.1ab	75.8±0.0a
Initial DTT	787.0±15.9bc	305.0±9.1cd	482.0±12.2b	706.0±6.8cd	401.3±11.3cd	4.1±0.1b	75.0±0.1a
PICS-oxy no DTT	475.7±29.7e	416.7±12.5bc	59.0±17.1c	890.0±54.1bc	473.3±42.1bc	5.0±0.4a	75.3±1.3a
PICS-oxy DTT	766.0±51.2bc	165.7±53.7e	600.3±6.7a	375.0±7.1e	276.7±63.6d	3.9±0.1b	73.3±0.1a
PICS-noxy no DTT	663.7±26.2cd	603.0±35.7a	60.7±13.3c	1257.0±72.2a	654.0±44.6a	5.2±0.5a	73.8±2.8a
PICS-noxy DTT	955.7±18.0a	303.7±11.7cd	652.0±23.4a	715.3±21.2cd	411.7±9.6cd	4.0±0.0b	73.7±0.5a
Woven no DTT	639.0±89.6cd	540.7±81.2ab	98.3±18.4c	1150.7±153.7a	610.0±72.5ab	4.6±0.2ab	75.8±0.0a
Woven DTT	900.3±40.6ab	250.7±10.0de	649.7±30.7a	608.3±15.0de	357.7±6.7dc	3.9±0.0b	73.7±0.5a

White maize

Treatment	PV	T	BD	FV	SB	PT	PTemp
Initial no DTT	1050.3±36.4a	998.3±43.0a	52.0±17.5c	2024.7±89.8a	1026.3±47.0a	5.5±0.5a	77.0±0.4a
Initial DTT	933.0±22.5c	437.3±30.0d	495.7±13.6a	909.3±55.0d	472.0±25.2c	4.2±0.0b	75.5±0.4b
PICS-noxy no DTT	887±0±41.0c	790.0±62.2b	97.0±21.2b	1639.0±162.6b	849.0±100.4b	5.1±0.5a	75.4±0.5b
PICS-noxy DTT	957.5±33.1bc	430.7±44.0d	526.7±11.1a	978.3±85.6cd	547.7±41.7c	4.2±0.1b	75.0±0.1b
Woven no DTT	1045.0±19.3ab	990.0±21.7a	55.0±5.2bc	1964.3±63.1a	974.3±82.4ab	4.9±0.2ab	74.8±0.5b
Woven DTT	1081.3±37.0a	547.7±21.0c	533.7±18.6a	1174.0±51.8c	626.3±31.6c	4.2±0.0b	75.0±0.1b

Means with different letters within the column for each genotype are significantly different Tukey test $p < 0.05$. Initial no DTT = initial flour DTT added to cooking water. Initial DTT = initial flour DTT added; PICS-oxy no DTT = flour from PICS bags with oxygen scavengers no DTT added to cooking water; PICS-oxy DTT = flour from PICS bags with oxygen scavengers DTT added; PICS-noxy no DTT = flour from PICS bags no oxygen scavengers without DTT added to cooking water; PICS-noxy DTT = flour from PICS bags without oxygen scavengers DTT added. Woven no DTT = flour from maize stored in woven bags no DTT added to cooking water; Woven DTT = flour from woven bags DTT added. PV; peak viscosity, FV; final viscosity, SB; setback, BD; breakdown, PT; peak time; PTemp; pasting temperature

Supplementary Table 2. Pasting profiles of flours treated with or without DTT after storage for 8

OPVI genotype

Treatment	PV	T	BD	FV	SB	PT	PTemp
Initial no DTT	430.3±19.0d	399.3±12.3b	31.0±19.2b	866.7±38.4bc	467.3±26.7ab	4.8±0.3bc	77.8±0.6abc
Initial DTT	559.0±0.0bcd	140.0±8.9d	419.0±10.2a	396.5±5.1d	257.0±8.8d	4.0±0.0c	75.0±0.0abcd
PICS-oxy no DTT	535.5±29.0bcd	505.0±19.8a	30.5±9.2b	1060.0±55.2a	555.0±35.4a	5.9±0.4ab	77.4±0.1abcd
PICS-oxy DTT	646.0±57.0abc	216.3±15.9cd	429.7±41.0a	531.0±30.8d	314.7±15.0cd	4.0±0.0c	73.7±0.5cd
PICS-noxy no DTT	487.0±39.0dc	467.3±32.9ab	19.7±7.0b	1007.3±86.4ab	540.0±53.7ab	6.7±0.2a	78.0±1.8ab
PICS-noxy DTT	748.0±11.4a	237.7±42.6c	510.3±31.2a	565.0±96.6d	327.3±54.1cd	4.0±0.1c	73.4±0.1d
Woven no DTT	424.7±31.7d	400.3±23.1b	24.3±11.0b	826.7±24.0c	426.3±13.4bc	5.9±1.1ab	78.3±2.2a
Woven DTT	649.3±90.4ab	203.3±26.0cd	446.0±65.2a	493.3±42.7d	290.0±20.7d	4.0±0.1c	73.9±0.6bcd

OPVII genotype

Treatment	PV	T	BD	FV	SB	PT	PTemp
Initial no DTT	594.0±37.7c	502.7±36.5ab	91.3±8.1c	1069.3±56.6ab	556.7±23.4ab	4.7±0.1b	75.8±0.0ab
Initial DTT	787.0±9.2b	305.0±9.1c	482.0±12.1b	706.0±6.8c	402.0±11.3c	4.1±0.1bc	75.0±0.1abc
PICS-oxy no DTT	588.3±31.4c	562.0±35.7a	26.3±6.7c	1186.3±63.7a	624.3±30.9a	5.5±0.3a	76.6±0.1a
PICS-oxy DTT	837.0±42.1ab	257.0±16.9cd	580.0±40.8a	591.7±31.8c	335.7±20.5c	3.9±0.1c	73.4±0.9c
PICS-noxy no DTT	474.5±6.4d	441.5±3.5b	33.0±2.8c	958.5±7.8b	517.0±4.2b	5.5±0.1a	75.9±0.0ab
PICS-noxy DTT	860.5±24.7ab	283.0±11.3c	577.5±13.4a	624.5±26.2c	341.5±14.8c	4.0±0.0c	73.9±0.6bc
Woven no DTT	242.5±3.5e	183.0±5.7d	59.5±2.1c	386.0±33.9d	203.0±28.4d	4.1±0.1bc	76.6±0.0a
Woven DTT	901.5±9.2a	319.0±0.0c	582.5±9.2a	724.5±6.4c	405.5±6.4c	4.1±0.0bc	73.8±0.6bc

White Maize genotype

Treatment	PV	T	BD	FV	SB	PT	PTemp
Initial no DTT	1050.3±36.4a	998.3±43.0a	52.0±17.5cd	2024.7±89.8a	1026.3±47.0a	5.5±0.5a	77.0±0.4a
Initial DTT	933.0±22.5b	437.3±30.0c	495.7±13.6a	909.3±55.0c	472.0±25.2c	4.2±0.0cd	75.5±0.4b
PICS-noxy no DTT	749.3±42.2c	461.7±40.1c	81.0±9.6c	1386.0±88.0b	717.7±47.8b	4.8±0.1bc	76.9±0.4a
PICS-noxy DTT	700.0±28.0c	230.0±20.2d	470.0±10.4a	553.7±34.8d	323.7±14.6d	4.0±0.1d	74.8±0.5b
Woven no DTT	492.0±45.5d	668.3±40.9b	30.3±6.3d	927.0±98.2c	464.7±59.7c	5.1±0.2ab	77.3±0.0a
Woven DTT	548.0±21.4d	211.0±9.2d	337.0±14.0b	499.0±12.5d	288.0±3.6d	4.1±0.1d	75.3±0.4b

Means with different letters within the column for each genotype are significantly different Tukey test $p < 0.05$. Initial no DTT = initial flour no DTT added to cooking water. Initial DTT = initial flour DTT added; PICS-oxy no DTT = flour from PICS bags with oxygen scavengers no DTT added to cooking water; PICS-oxy DTT = flour from PICS bags with oxygen scavengers DTT added; PICS-noxy no DTT = flour from PICS bags no oxygen scavengers without DTT added to cooking water; PICS-noxy DTT = flour from PICS bags without oxygen scavengers DTT added. Woven no DTT = flour from maize stored in woven bags no DTT added to cooking water; Woven DTT = flour from woven bags DTT added. PV; peak viscosity, FV; final viscosity, SB; setback, BD; breakdown, PT; peak time; PTemp; pasting temperature.

APPENDIX B. PUBLISHED ABSTRACT AND PUBLICATION

Abstract

Reducing carotenoid degradation is key to maintaining nutritional quality of high carotenoid biofortified maize during storage

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Abstract

Provitamin A carotenoid (PVAC) rich biofortified maize holds promise as a means to address vitamin A deficiency in developing countries. However, carotenoid degradation during post-harvest storage can result in significant reduction of PVAC. In this study, we assessed the use of Purdue Improved Crop Storage (PICS) bag to slow oxidative degradation of carotenoids. PICS bags are a low-cost storage system designed to provide a hermetically sealed oxygen deprived environment to control pests in stored grains. We hypothesized that maize stored in oxygen reduced PICS bags would further reduce carotenoid degradation and provide a higher nutritional quality maize. Freshly harvested grain from two biofortified maize genotypes, OPVI and OPVII, was dried to a moisture content of ~8.5% and put in storage for 8 months in PICS-oxy (oxygen scavenging), PICS-noxy (no scavenging) and woven bags each at 50 kg/bag at temperature ~29ºC and ~30%rh. Carotenoid content was determined by liquid chromatography at 4 and 8 months and % recovery was calculated by comparing individual and total carotenoid (TC) contents at each time point to the initial content. Initial carotenoid contents (μg/g DW) of OPVI

vs OPVII were 37.0 vs 22.7 (zeaxanthin), 7.2 vs 11.4 (lutein), 2.8 vs 2.7 (*β*-cryptoxanthin), 2.5 vs 3.2 (*trans*-*β*-carotene) and 3.5 vs 3.9 (*cis*-*β*-carotene). After 4 months, OPVI (76, 51, 56%) had significantly higher retention ($p<0.05$) for PVAC than OPVII (57, 47, 46%) and TC retention in OPVI (90, 73, 71%) was significantly higher than OPVII (76, 69, 64%) in PICS-oxy, PICS-noxy and woven, respectively. PICS-oxy had higher retention than PICS-noxy and woven bags, indicating both genotype and storage bag had a significant effect on carotenoid stability confirming the importance of entrapped oxygen and oxidation as the likely primary mechanism of carotenoid degradation in storage. After 8 months, retention of PVAC was similar but higher in OPVI (45, 44, 38%) than OPVII (34, 34, 34%) and TC retention for OPVI (63, 48, 51%) was higher than OPVII (51, 49, 43%) in PICS-oxy, PICS-noxy and woven, respectively. In conclusion, stability of carotenoids during post-harvest storage is dependent on low oxygen content, genotype and storage period. Furthermore, the PICS storage system can be an effective method capable of managing oxygen and carotenoids stability.

https://academic.oup.com/cdn/article-pdf/2/11/nzy030/26908531/nzy030.pdf

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Publication

Nkhata SG, Ortiz D, Baributsa D, Hamaker B, Rocheford T, Ferruzzi MG. Assessment of oxygen sequestration pn effectiveness of Purdue Improved Crop Storage (PICS) bags in reducing carotenoid degradation during post-harvest storage of two biofortified orange maize genotypes. J Cereal Sci. 87, 68-77. https://doi.org/10.1016/j.jcs.2019.02.007

APPENDIX C. STANDARD OPERATING PROCEDURES

Extraction of c arotenoids derivatives from cereals

1. Keep ground sample or porridge on ice while weighing and covered.
2. Weigh 500 mg of ground maize flour in a 1 5 ml Falcon tube, polypropylene. Keep weighed samples on regular ice in covered Styrofoam cooler. Keep covered to reduce light.
3. Spike : Spike samples at this point with 80 µl of β -apo -8-carotenal (resolubilize in 1ml Petroleum ether). Run 2 spikes and one control samples for each extraction set. Also run 3x spikes only, inject 10uL.
4. Add 1.0 ml of DI water. Vortex to mix. Place on ice in covered cooler for 10 minutes.
5. Add 5 ml of Chilled Acetone. Vortex to mix. Agitate for 5 minutes on rotary mixer. Wrap sample rack in foil to reduce light. Incubate for 5 minutes on ice in covered ice cooler.
6. Centrifuge samples: runs the centrifuge at 3000 RPM for 10 minutes. Centrifuge is cooled to 4 C.
7. Remove samples from centrifuge and transfer acetone into a second 15 ml polypropylene falcon tube.
8. Place acetone fraction under nitrogen.
9. Add a 5 ml aliquot of chilled acetone to grain sample. Vortex to mix pellet. Agitate for 5 minutes on rotary mixer. Incubate for 10 minutes instead of 5 minutes on ice. Centrifuge. Add this second acetone fraction to the initial acetone fraction under nitrogen.
10. Add 2 ml of MTBE to ground sample. Vortex to mix pellet. Agitate for 5 minutes on rotary mixer. Incubate for 10 minutes on ice in covered Styrofoam cooler. Vortex one more time.
11. Centrifuge samples: runs the centrifuge at 3000 RPM for 5 minutes. Centrifuge is cooled to 4 C.
12. Add the MTBE fraction to the Acetone fractio ns dry under nitrogen.
13. Redissolve* in 2mL of Ethyl acetate:Methanol (1:1) for immediate analysis. Vortex briefly to mix sample and syringe filter through 0.45mm filters.
14. Run in HPL, using "C30SHORT" method on HP1090. Inject 10uL. Any unused material should be kept in the freezer. Beta Carotene is sensitive to light and heat. Care should be taken to keep ground samples in low light conditions as much as possible.

Porridge preparation
Ferruzzi Lab Pr otocol updated 10/14/11

1. Prepare slurry (10g of maize flour + 20mL of distilled water).
2. Boil 20mL of distilled water.
3. Add slurry to boiling water, use 5mL of water to rinse all the flour into the boiling water, stir the mixture on an electronic hot plate for 5 min.
4. Set the porridge under foil at room temperature for 10min.
5. Weigh the porridge in tubes need for each analysis.

Flush with N2 and Freeze sample s

Extraction of Carotenoids derivatives from maize porridge

1. Keep porridge on ice while weighing and covered.
2. Weigh 2g of maize porridge in a 1 5 ml Falcon tube, polypropylene. Keep weighed samples on regular ice in covered Styrofoam cooler. Keep covered to reduce light.
3. Spike : Spike samples at this point , with Spike 80uL β-apo -8-carotenal (resolubilize in 4 mL Petroleum ether) . Run 2 spikes and one control samples for each extraction set. Also run 3x spikes only, inject 10uL.
4. Add 5 ml of Chilled Acetone. Break up large chunks with spatula. Vortex to mix. Agitate for 5 minutes on rotary mixer. Wrap sample rack in foil to reduce light. Incubate for 5 minutes on ice in covered ice cooler.
5. Centrifuge samples: runs the centrifuge at 3000 RPM for 10 minutes. Centrifuge is cooled to 4 C.
6. Remove samples from centrifuge and transfer acetone into a second 15 ml polypropylene falcon tube.
7. Place acetone fraction under nitrogen.
8. Add a 5 ml aliquot of chilled acetone to grain sample. Vortex to mix pellet. Agitate for 5 minutes on rotary mixer. Incubate for 20 minutes instead of 5 minutes on ice. Centrifuge. Add this second acetone fraction to the initial acetone fraction under nitrogen.
9. Add 2 ml of MTBE to sample. Vortex to mix pellet. Agitate for 5 minutes on rotary mixer. Incubate for 20 minutes on ice in covered Styrofoam cooler. Vortex one more time.
10. Centrifuge samples: runs the centrifuge at 3000 RPM for 10 minutes. Centrifuge is cooled to 4 C.
11. Add the MTBE fraction to the Acetone fractions dry under nitrogen.
12. Add 2ml of MTBE to sample. Vortex to mix. Agitate for 5 minutes on rotary mixer. Centrifuge. Add this second MTBE fraction to the initial MTBE fraction under nitrogen.
13. Resolubilize 500uL ethyl acetate and 500uL methanol for immediate analysis. Vortex briefly to mix sample and syringe filter through 0.45mm filters.
14. Run in HPL, using "C30SHORT" method on HP1090. Inject 10uL. Any unused material should be kept in the freezer. Beta Carotene is sensitive to light and heat. Care should be taken to keep ground samples in low light conditions as much as possible.

Three Stage In vitro Digestion for Porridge

Ferruzzi Lab Protocol updated 11/15/2010

Preparation

Stock Solutions:

0.9% NaCl

Gastric and Small Intestinal Phase Solutions:

Pepsin Solution **(2mL per reaction)**

100mM $NaHCO_3$
1.0 M HCl
0.1M HCl
1.0M NaOH
0.1M NaOH

10 mg/mL Pepsin in 0.1M HCl
Pancreatin-Lipase Solution **(2mL per reaction)**
20 mg/mL Pancreatin (in 100mM $NaHCO_3$)
10 mg/mL Lipase (in 100mM $NaHCO_3$)
Bile Solution **(3mL per reaction)**
30 mg/mL Bile Extract (in 100mM $NaHCO_3$)

Other Materials: 50 mL tubes, 15mL tubes, 1.0, 5.0 mL Pipetter, pH meter, shaking water bath, N_2 tank.

Preparation of Oral Phase:
Base Solution (q.s. to 1 L with Dl water):
Potassium Chloride 1.792g
Sodium Phosphate 1.776g
Sodium Sulfate 1.140g
Sodium Chloride 0.596g
Sodium Bicarbonate 3.388g

For ~10 g porridge

Prep 100 mL oral phase solution
-Need 6 mL per digestion, but hard to measure small amounts

1. Add 100 mL base solution to beaker with stir bar
2. Add 40 mg urea
3. Add 3 mg uric acid
4. Add 5 mg mucin per **mL** base solution.
5. Add 3.18 g α-amylase
6. Mix well (at least 15 minutes)

α-amylase –Sigma, A3176. The activity is 15.8 units/mg of solid at pH 6.9 of food to be digested
(15.8 units/mg) x (31.8 mg/ml) x (6ml) = 3015 units per digestion

OPM = oscillations per minute

Final Concentrations:
Pepsin = 0.5 g / L = (10 mg/mL x 2 mL) / 40 mL
Pancreatin = 0.8 g/L = (20 mg/mL x 2 ml) / 50 mL
Lipase = 0.4 g/L = (10 mg/mL x 2 mL) / 50 mL
Bile = 1.8 g/ L = (30 mg/mL x 3 mL) / 50 mL

Procedure:

Start Up.

1. Weigh 10 g of porridge to be digested into a 50 mL centrifuge tube (in triplicate).
2. Collect 3x10 mL aliquots of porridge as **Raw Material (RM)**, freeze. Freeze remaining porridge.

Oral Phase

3. Add 6 mL oral phase per reaction tube (see previous for preparation of oral phase)
   Vortex or homogenize depending on food matrix (1 minute).
4. Blanket with nitrogen gas, cap tightly, and seal with parafilm.
5. Place horizontally in 37 °C water bath. Shake at 85 opm for **10 minutes.**
6. **Meanwhile, prepare pepsin solution**

Gastric Phase

7. Remove from water bath, place immediately on ice.
8. Bring to 30 mL with saline. (Assume 1g food material = 1mL)
9. Adjust pH to equal 3.5±0.1 using 1.0 N HCI. Measure in increments of 0.1 and 0..5 mL recording pH and volume after every addition.
10. Add *2* mL 10 mg/mL pepsin
11. Adjust pH to equal **2.5** ±0.1 using 1.0 N HCI.
12. Bring to 40 mL with Saline
13. Blanket with nitrogen gas, cap tightly and place horizontally in 37 °C Water Bath.
14. Incubate at 90 opm for **1 hr**.
15. Meanwhile, prepare bile extract. Sonicate for 30 minutes. 45 minutes into incubation, prepare pancreatin-lipase solution.

Intestinal Phase

16. Remove from water bath, place immediately on ice.
17. Adjust pH to equal pH 5.0 ± 0.1 1 N $NaHCO_3$. Record volume and pH at each step.
18. Add 2 mL 20mg/mL Pancreatin (final concentration = 0.8 g/L) + 10mg/mL Lipase(final concentration = 0.4 g/L)
19. Add 3 mL 30 mg/mL Bile extract Solution (final concentration = 1.8 g/L)
20. Adjust pH to equal **pH 6.5** ± 0.1 1 N $NaHCO_3$. Record volume and pH at each step.
21. Bring to 50 mL with saline.
22. Blanket with nitrogen gas, cap tightly and place horizontally in 37 °C Water Bath.
23. Incubate at 90 opm for **2 hr**.

Isolation of Micellar Fraction

24. Remove from water bath and collect 3 x 5 mL aliquot of finished **Digesta (DG)** in 15mL tube, flush with N_2 and freeze.
25. Transfer 30 mL of digesta to appropriate polycarbonate or polyallomar tubes for either Ultracentrifugation (167,000 xg for 95 min) or High Speed Centrifugation (10,000 xg for 1h).
26. Following centrifugation collect 3 x 5 mL of **aqueous fraction (AQ)** and syringe filter through 0.22mm filters.

27. Collect remaining filtered **aqueous fraction** in 15mL tube, flush with N_2 and freeze.

Calculations

Digestive Stability = moles of compound in final DG / moles of compound in RM x 100

Micellarization Efficiency = moles of compound in final AQ / moles of compound in DG x 100

Carotenoids extraction from AQ/DG fraction from maize Porridge digestion

Ferruzzi Lab Protocol updated 10/14/11

1. Thaw 10mL aliquot of AQ or DG in cold water
2. Take 4 ml into 15mL tube (n=3)
3. Spike samples either AQ or DG using 80uL B-apo -8-carotenal
    - AQ samples spike dilution 1/5
    - DG samples spike dilution 1/20
4. Extract with 1mL acetone and 3mL petroleum ether (0.1% BHT).
5. Vortex samples for 1min
6. Centrifuge 2min at 3.5 rpm
    - Add 150 uL isopropanol if needed for break cloudy layer
7. Place on ice and transfer organic layer to culture tube
8. Repeat acetone and petroleum ether extraction 2 times
9. Dry under nitrogen
10. Resolubilize
    - AQ samples in 150uL (75uL ethyl acetate:75uL methanol)
    - DG samples in 600uL (300uL ethyl acetate: 300uL methanol enough to dilute residual oil/lipids)
11. Transfer to 2mL Eppendorf tube
12. Centrifuge for 5 min at 14000rpm
13. Inject :
    - Control salad 10uL
    - AQ/DG samples 20uL