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Steeping of Biofortified Orange Maize Genotypes for Ogi Production Modifies Pasting Properties and Carotenoid Stability

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Received: 30 September 2019; Accepted: 30 October 2019; Published: 18 November 2019

Abstract: Biofortified orange maize open-pollinated varieties and hybrids with higher provitamin A carotenoids (pVACs) have been released in sub-Saharan Africa and will be introduced throughout the local food systems. This study assessed the impact of steeping, a traditional processing method, on retention of carotenoids and starch pasting properties of porridges made from select biofortified maize genotypes. Steeping had a modest effect (<9% loss) on total carotenoid stability during relatively shorter steeping periods (<72 h). However, more extended steeping periods (up to 120 h) had a detrimental effect on total carotenoid recovery (61% loss). Xanthophylls showed greater stability (82% retention) compared to carotenes (30% retention) during subsequent wet cooking of fermented flours. Interestingly, steeping of maize did modify pasting properties, with peak viscosities increasing from 24–72 h of steeping potentially impacting cooking stability. These results suggest that steeping can impact carotenoid retention and potentially optimal steeping times would be 24–72 h for acceptable carotenoid retention.

Keywords: biofortified maize; pro-vitamin A; lutein; zeaxanthin; carotenoids; degradation; retention; steeping; fermentation; food processing; nutritional impact

Chemical Compounds Studied in This Article:

β-Carotene (PubChem CID: 5280489); Zeaxanthin (PubChem CID: 5280899); Lutein (PubChem CID: 5368396); β-cryptoxanthin (PubChem CID: 182237)

1. Introduction

Vitamin A deficiency (VAD) continues to be a significant health concern in developing countries affecting more than 190 million preschool-age children and 19.1 million pregnant women globally [1]. Blindness, anemia, and infant mortality are all consequences of VAD that impact the quality of life in affected regions. Aggressive and transformative strategies are needed to overcome this nutritional challenge. A major factor in VAD is the lack of dietary vitamin A or provitamin A carotenoids (pVAC) in staple foods. Biofortification of grain-based staple foods including maize, sorghum, and rice with pVACs has been touted as a promising approach to address this issue directly for broader

portions of affected populations. Biofortified maize (*Zea mays L.*) genotypes have been developed through conventional breeding programs that produce yellow and orange kernels with high contents of β-carotene and other pVACs [2,3]. Success in terms of biofortification of maize includes a target level of 15 μg/g of β-carotene, to provide an ~50% of the Estimated Average Requirement (EAR) for vitamin A in maize-eating regions [4,5]. Included in this EAR estimates are that these levels should be retained through post-harvest and food processing [6,7] and considers ultimate bioavailability in humans [8–11].

With breeding efforts now generating elite genotypes with >20 μg/g of total pVACs, downstream processing and bioavailability are becoming critical factors to study. Losses during postharvest handling (drying, milling, and storage) and food processing can be significant as pVACs are sensitive to heat, oxygen, light, and acidic conditions [12]. In African countries, maize and other cereals are commonly processed by steeping, in which natural fermentation is allowed to occur by soaking cereals in water spontaneously. Additionally, maize is utilized in several common preparations including bread, beverages, and porridges known such as ogi (Nigeria/West Africa), kenkey (Ghana), uji (Kenya), togwa (Tanzania), amahewu (South Africa), and mawé (Benin) [13]. These spontaneously fermented products are dietary staples for adults and children alike [14]. Most of these fermentations are spontaneous and rely on native lactic acid bacteria, sometimes accompanied by yeast fermentation [14]. The fermentation process can improve nutrient quality, and density, and increase the bioavailability of certain nutrients in foods [15,16]. For example, fermentation of grains has been associated with increased starch and protein digestibility [17], as well as an increase in lysine and phenolics bioavailability [18,19]. Organic acids produced during fermentation have been reported to enhance iron and zinc bioavailability through the formation of soluble ligands [20]. Fermentation also lowers the pH which can favorably affect activity of endogenous phytase, resulting in lower phytic acid [21]. These efforts combined suggest that fermentation of grains has the potential to reduce the risk of mineral deficiency among populations, especially in developing countries where unrefined cereals and pulses are highly consumed [22].

As fermentation of maize for porridge production is commonly practiced throughout sub-Saharan Africa, it is critical to developing a better understanding of its impact on carotenoid stability, and potential bioavailability is needed. Carotenoids in starchy staple crops are believed to be minimally affected during fermentation. Thakkar et al. [23] reported that β-carotene retention was 92% after three days fermentation of grated cassava, followed by 63% retention after roasting (165 °C, 10 min). A similar study on transgenic cassava roots carried out by Failla et al. [24] reported that fermentation followed by roasting resulted in less than 40% retention of β-carotene. Recently, Aragón et al. [25] working with an elite selection of biofortified cassava roots reported that spontaneous fermentation decreased levels of total carotenoid content (TCC) and β-carotene equivalent, with retentions ranging from 72% to 96% among ten genotypes.

While promising, less has been reported from biofortified maize grains. Li et al. [26] studied the effect of spontaneous fermentation at room temperature (30 °C) in the dark for 48 h (solid-state fermentation) as part of the preparation of Ogi, a fermented maize porridge, made from high pVACs maize inbreeds. An initial 7% β-carotene loss was observed during 24 h soaking and milling, followed by an additional 10% loss during spontaneous fermentation, and a 7% loss during cooking.

Considering these findings, it is critical to include other elite biofortified maize genotypes as well as considering the potential impacts on product properties that may relate to carotenoid bioavailability and texture qualities [27]. The texture of the final product is critical to the acceptability for consumers of biofortified fermented porridge. Textural qualities of Ogi depend on many factors, which include maize genotype, maize flour particle size, fermentation, and souring periods [28]. Starch and its biochemical characteristics related to the ratio of amylose and amylopectin influence the viscosity and gelatinization properties of the starch, which impact the final texture and sensory attributes of typical maize-based foods [29]. The characteristic of the starch granule, such as swelling, breakdown, and retrogradation, largely determine the overall pasting properties and stability of starchy foods [30]. Establishing the impact of critical processes such as fermentation on the recovery of pVACs while

simultaneously selecting rheological properties of the biofortified maize flour is critical to realizing the potential of these biofortified grains by facilitating their transfer to consumer foods. With this in mind, the objective of this study was to assess the impact of spontaneous fermentation on carotenoid stability and pasting properties of finished flours from five selected elite biofortified maize genotypes.

2. Materials and Methods

2.1. Chemicals and Standards

Extraction and HPLC solvents including acetone, ethyl acetate, methanol, petroleum ether (JT Baker, Phillipsburg, NJ, USA), and methyl tert-butyl ether (Sigma-Aldrich, St. Louis, MO, USA) were all certified HPLC and ACS-grade. A 1.0 mol L^{-1} solution of ammonium acetate (Sigma-Aldrich) was made using double distilled water and adjusted to pH 4.6 with glacial acetic acid. All-trans standards of lutein (LUT), β-carotene (BC), β-cryptoxanthin (BCRYP), β-apo-8′-carotenal (Sigma-Aldrich, St. Louis, MO, USA), α-cryptoxanthin, α-carotene (CaroteneNature, Lupsingen, Switzerland), and zeaxanthin (ZEA; IndoFine, Hillsborough, NJ, USA) were obtained and used for carotenoid identification and calibration of the HPLC method.

2.2. Biofortified Yellow and Orange Endosperm Maize Genotypes

Five experimental maize (*Zea mays*) genotypes were chosen based on their unique carotenoid profiles and different kernel textures. The genetic pedigrees of the five maize genotypes are provided in Table 1. Genotypes 1, 2, and 5 are the same genotypes reported on by Ortiz et al. [6]. Genotypes 3 and 4 are from a recurrent selection of the same open-pollinated variety but different cycles and years. These kernels were stored for two years at −20 °C before use in this study. The absolute values of carotenoid content reported for these genotypes, therefore, did not reflect the same precise carotenoid levels at harvest point, yet the relative carotenoid levels of the genotypes tended to remain consistent under −20 °C storage conditions. More detailed information on pedigree and growing conditions of the different genotypes used in this study is reported in Ortiz et al. [6].

Table 1. Genetic pedigree of the genotypes evaluated.

Genotype	Pedigree
1	C17 × DE3
2	Hi27 × CML328
3	2013 Orange ISO
4	2015 Orange ISO
5	[KUI carotenoid syn-FS17-3-1-B-B-B-B-B-B-B-B] × [(MAS[206/312]-23-2-1-1-B-B-B/[BETASYN]BC1-6-5-1xFloridaASYN#-B)-B-1-3-B-B-B]

2.3. Processing Method of Maize

Ogi, a traditional African fermented porridge processed by steeping whole grains, was prepared using each biofortified maize genotype according to the procedure reported by Aremu [31] with slight modifications. For each genotype, ~100 g kernels in triplicate were steeped in 200 mL deionized water in quarter pint mason jars. Although maize kernels absorbed water during steeping, the amount of water used in the experiment was enough to keep all maize kernels submerged under the water, with a least a 1.5 cm water layer above them, during the entire steeping period. Jars were tightly closed with a 2 cm air headspace and allowed to stand in a room with controlled temperature (27 °C) in a dark condition to minimize photo-isomerization reaction for 24, 72, and 120 hours of steeping time. Steeping time is the time where natural fermentation is allowed to occur by soaking the maize kernels in water spontaneously. The temperature was chosen on the basis of the practical availability of a controlled-temperature storage condition. Next, the steeping water was decanted, and the water

pH measured. Each sample was then milled with the steeping water for 5 min at high speed with an Osterizer® 10 speed blender (Sunbeam Products Inc, Boca Raton, FL, USA). The resulting slurry was then filtered with a cheesecloth #90 by hand and squeezed until the water was no longer released from the residue. The resulting material was dried using a freeze-dryer system (SP Scientific, Model #50L Virtual EL-85, Warminster, PA, USA) over a three days period to a final moisture content of 10.5% ± 8.1%. Finally, samples were milled to <0.5 mm with a Foss CT 1093 Cyclotec^{TM} sample mill (Foss Analytical Co, Suzhou, China).

2.4. Biofortified Maize Porridge Preparation

Test porridges were prepared using both non-fermented and fermented biofortified maize, as a starting material as described by Lipkie et al. [32]. Briefly, a slurry of 10 g of fine maize meal (either fermented or unfermented) and 20 mL of distilled water were added to 20 mL of boiling distilled water, stirred by hand with a spatula for 5 min, covered with foil, allowed to sit for 30 min at ambient temperature, and then stored at −80 °C until analysis. The dry matter content for this formulation was 27.0 ± 2.6%.

2.5. Assessment of Pasting Properties by Rapid Visco Analyser (RVA)

Pasting properties for the native and steeped whole-maize flours for each genotype was investigated using a rapid visco-analyzer (RVA) model RVA-4 series (Newport Scientific, Warriewood, Australia) following the procedure reported by the American Association of Cereal Chemist method (76-21.01) with minor modifications. Briefly, 8% hot flour dispersions (W/V distilled water) were prepared and the viscosity was recorded using the following temperature profile: holding at 50 °C for 1 min, heating from 50 to 95 at 12 °C/min, holding at a 95 °C plateau for 2 min, and then cooling down to 50 at −12 °C/min with continuous stirring at 160 rpm. Six parameters were reported from the viscoelastograms: pasting temperature, which is the temperature when the viscosity of the starch begins to rise; peak viscosity (PV), the highest viscosity reached during heating or pasting and is reached at the end of the heating stage when the high number of swollen starch granules results in pasting; hot paste viscosity or holding strength (HPV, lowest hot paste viscosity), measures the viscosity when the swelled starch granules were disrupted upon shearing and heating; cold paste viscosity at 50 °C (CPV), measures the ability of the starch to form viscous paste after cooking and cooling; breakdown viscosity (BS, estimated as PV-HPV), explains the hydration, starch swelling power and shear resistance of starch paste during heating; and setback (SB, estimated as CPV-PV), indicates starch retrogradation tendency after gelatinization and cooling at 50 °C [33].

2.6. Carotenoid Analysis

All sample preparations and extractions were performed under yellow light to minimize the potential for photo-isomerization reactions. Carotenoid extractions were performed using the procedure described by Ortiz et al., [34] with minor modification. Briefly, ground maize flours (0.6 g) or porridge (2 g) were spiked with 80 µL internal standards (150 µM or 30 µM β-apo-8'-carotenal in ethyl acetate). Ground maize flours were hydrated with 1 mL of distilled water on ice for 10 min, and carotenoids were extracted twice with 5 mL of cold acetone and once with 2 mL of methyl tert-butyl ether. Extracts were dried under a stream of nitrogen, resolubilized in 1:1 methanol:ethyl acetate, filtered through a 0.45 µm PTFE syringe filter (Macherey-Nagel, Düren, Germany) and then analyzed by HPLC. Carotenoids were separated using a YMC C30 (3 µm 2.0 mm × 150 mm column), with a YMC carotenoid guard column (2.0 mm × 23 mm) in a Hewlett-Packard 1090 HPLC system coupled with a diode array detector at 450 nm as previously described by Ortiz et al. [34]. A gradient elution profile based on a binary mobile phase system consisting of Methanol:1 M ammonium acetate (98:2 v/v) in phase A and ethyl acetate in phase B was used. A flow rate of 0.37 mL/min was utilized with initial conditions set at 0 min 80:15 v/v, phase A:phase B; 6 min 20:80 v/v, phase A:phase B; 8 min 0:100 v/v, phase A:phase B; 12 min 0:100 v/v, phase A:phase B, 14 min 80:15 v/v, phase A:phase B. Peaks were identified by comparing spectral

information in the literature [35] and retention times with authentic all-trans-carotenoid standards. Quantification was based on seven-point calibration curves prepared spectrophotometrically with authentic all-trans-standards with a concentration range between 0.01 to 7.67 μM.

2.7. Statistical Analysis of Data

All data were expressed as mean ± standard error of the mean (SEM) with a minimum of triplicate sample extraction and were analyzed for statistically significant differences (ANOVA) using JMP 12.0.1 (SAS Institute, Cary, NC, USA) using the Tukey−Kramer method for pairwise comparison. A completely randomized two-factor factorial design was used to investigate the impact of genotype and fermentation time on carotenoid stability simultaneously. A mixed-design analysis of variance model was used to test the retention of carotenoid species among genotypes within the steeping period. An ANOVA model was used to explore the main effect of carotenoid species and genotype on carotenoid retention and its respective interaction (carotenoid species × genotype). Total carotenoid content was calculated by the summary of each individual carotenoid identified and quantified by analysis. Carotenoid retention was calculated by the following mathematic formula: 100 – ((initial concentration-final concentration)/(initial concentration)) × 100. Provitamin A content was defined in β-carotene equivalents, which is equal to all-trans-β-carotene + $\frac{1}{2}$ (β-cryptoxanthin + α-carotene + cis-β-carotene isomers).

3. Results

3.1. Carotenoid Profile on Selected Biofortified Maize Genotypes

Biofortified maize kernels used in this experiment contained 32.7–61.0 μg/g total carotenoids, dry weight basis (DW; Table 2). Xanthophylls, including LUT and ZEA, were the primary carotenoid forms among the different genotypes and represented 66%–91% of total carotenoid content averaged over genotypes. Zeaxanthin was the major xanthophyll among the different genotypes evaluated and represented 55% (11%–71%) of total carotenoid content in all maize samples. The subsequent major carotenoid was LUT, representing 23% (14%–52%) of total carotenoid content. Notably, for genotype C17 × DE3, ZEA and LUT represented ~11% and ~52% of the total carotenoid content, respectively. Provitamin A carotenoids in β-carotene equivalents were found to range from 4.3–9.3 μg/g DW, and the relative proportion of pVACs in relation to total carotenoids ranged from 9% to 28% across genotypes evaluated. The 2013 Orange ISO genotype contained the highest ($p < 0.05$) concentration of total carotenoids (61.0 μg/g DW) across all genotypes. In contrast, C17 × DE3 had the lowest concentration of total carotenoids among all genotypes evaluated (32.7 μg/g DW) yet showed the highest concentration of pVACs (9.3 μg/g DW). The main pVAC compounds found among the maize genotypes analyzed were BC and BCRYP, these compounds represented ~9% (4%–21%) and ~6% (2%–9%) of the total carotenoid content, respectively. The C17 × DE3 genotype contained the highest amount of BC at 6.8 μg/g DW, and Hi27 × CML328 showed the highest levels of BCRYP at 5.3 μg/g DW.

Table 2. Carotenoid concentration (μg/g dry weight (DW)) after traditional African fermentation "ogi", after 0, 24, 72, and 120 h.

Fermentation (hours)	Genotype [1,2]				
	1	**2**	**3**	**4**	**5**
	all-trans-lutein				
0	17.1 ± 0.3a	8.1 ± 0.2a	8.9 ± 0.2a	7.5 ± 0.1a	7.8 ± 0.5a
24	16.3 ± 1.2a	7.8 ± 0.7a	8.6 ± 0.3a	6.4 ± 0.3b	7.1 ± 0.9a
72	17.2 ± 0.8a	7.1 ± 0.4a	7.8 ± 0.4b	5.8 ± 0.4b	8.9 ± 1.0a
120	6.6 ± 0.2b	2.7 ± 0.2b	5.2 ± 0.9c	2.2 ± 0.1c	3.3 ± 0.3b

Table 2. *Cont.*

Fermentation (hours)	Genotype [1,2]				
	1	**2**	**3**	**4**	**5**
all-trans-zeaxanthin					
0	3.7 ± 0.2a	34 ± 0.6a	43.3 ± 0.7a	33.5 ± 0.4a	29.2 ± 0.9a
24	3.7 ± 0.2a	32.7 ± 3.4b	45.5 ± 1.6a	30.0 ± 0.9a	20.3 ± 3.0b
72	3.9 ± 0.2a	31.3 ± 2.0b	39.1 ± 2.3b	26.5 ± 2.6b	24.8 ± 2.7b
120	1.5 ± 0b	11.5 ± 0.6c	20.6 ± 5.1c	9.9 ± 0.1c	9.0 ± 0.9c
β-cryptoxanthin					
0	0.8 ± 0.1a	5.3 ± 0.3b	3.0 ± 0.1a	2.2 ± 0.2a	3.6 ± 0.3a
24	0.8 ± 1.1a	7.7 ± 0.5a	4.0 ± 0.3a	2.3 ± 0.1a	3.5 ± 0.5a
72	0.8 ± 0.0a	6.4 ± 0.9b	3.1 ± 0.2a	2.1 ± 0.1a	4.0 ± 0.4a
120	0.4 ± 0.1a	2.6 ± 0.1c	1.6 ± 0.3b	0.8 ± 0.0b	1.5 ± 0.1b
all-trans-β-carotene					
0	6.8 ± 0.3b	4 ± 0.2a	2.8 ± 0.1a	2.1 ± 0.2a	2.6 ± 0.2a
24	7.1 ± 0.5ab	5.1 ± 0.4a	2.9 ± 0.3a	1.9 ± 0.1a	2.2 ± 0.4a
72	8.2 ± 0.3a	4.8 ± 0.6a	2.9 ± 0.2a	1.8 ± 0.2a	2.7 ± 0.3a
120	3.2 ± 0.2c	1.9 ± 0.1b	1.5 ± 0.3b	0.7 ± 0.0b	1.0 ± 0.1b
total carotenoid content					
0	32.7 ± 0.9a	55.9 ± 1.5b	61.0 ± 1.3ab	47.5 ± 0.9a	46.1 ± 2.2a
24	31.7 ± 2.0a	58.9 ± 3.3a	64.1 ± 2.7a	42.6 ± 1.4b	35.3 ± 4.8a
72	34.4 ± 1.3a	54.5 ± 3.7b	55.9 ± 3.1b	38.2 ± 3.3b	43.0 ± 4.7a
120	13.6 ± 0.4b	20.6 ± 1c	30.4 ± 6.9c	14.5 ± 0.1c	15.8 ± 1.5b
Xanthophylls					
0	21.6 ± 0.5a	47.4 ± 1a	55.2 ± 1.0ab	43.2 ± 0.6a	40.6 ± 2.2a
24	20.8 ± 1.4a	48.2 ± 2.5a	58.1 ± 2.2a	38.6 ± 1.2b	30.9 ± 4.9b
72	21.9 ± 1.0a	44.9 ± 3.2b	50.1 ± 2.9b	34.4 ± 3.0b	37.7 ± 4.1b
120	8.6 ± 0.2b	16.8 ± 0.9c	27.4 ± 6.3c	13.0 ± 0.1c	13.7 ± 1.4c
provitamin A carotenoids[3]					
0	9.3 ± 0.4a	8.9 ± 0.5a	5.9 ± 0.3a	4.3 ± 0.3a	5.8 ± 0.6a
24	9.4 ± 0.5a	11.8 ± 0.8a	6.4 ± 0.5a	4.0 ± 0.2a	5.1 ± 0.7a
72	10.8 ± 0.3a	10.4 ± 1.3a	5.9 ± 0.3a	3.8 ± 0.3a	6.0 ± 0.7a
120	4.3 ± 0.2b	4.1 ± 0.1b	3.0 ± 0.6b	1.5 ± 0.0b	2.2 ± 0.2b
sum of cis-β-carotene					
0	4.3 ± 0.2a	4.5 ± 0.3a	3.0 ± 0.3a	2.2 ± 0.1a	2.9 ± 0.4a
24	3.9 ± 0.2a	5.5 ± 0.4a	3.0 ± 0.4a	2.1 ± 0.1a	2.2 ± 0.3a
72	4.3 ± 0.1a	4.8 ± 0.6a	2.9 ± 0.1a	1.9 ± 0.1a	2.5 ± 0.3a
120	1.9 ± 0.1b	1.9 ± 0.1b	1.5 ± 0.3b	0.8 ± 0.0b	1.1 ± 0.1b

[1] Columns not connected by the same letter within each carotenoid species are significantly different according to Tukey's HSD test ($p < 0.05$). [2] All data are expressed as μg of carotenoids per g of maize food (dry weight). Carotenoid data are expressed as mean ± SEM; $n = 4$. [3] Provitamin A content in content in β-carotene equivalents = all-trans-β-carotene + $\frac{1}{2}$ (α-carotene + β-crytoxanthin + 15-cis-β-carotene + 13-cis-β-carotene + 9-cis-β-carotene).

3.2. Effect of Fermentation on Carotenoid Recovery in Biofortified Maize

Steeping of whole kernels significantly impacted the pH of the resulting flour ($p < 0.001$), with an average reduction from 6.8 to ~5.0 (4.9–5.2) after 24 h (Supplementary Table S1). The pH subsequently remained stable from 24 to 120 h. The steeping period significantly impacted carotenoid retention ($p < 0.001$) in biofortified maize kernels. Carotenoid stability through fermentation also showed a genotype effect ($p < 0.0001$). Individual carotenoid species stability during steeping/fermentation was not dependent on maize genotypes ($p > 0.05$), suggesting that the effect of steeping/fermentation on carotenoid retention was a generalized effect over all carotenoid isomers among the different

biofortified genotypes tested. The overall total carotenoid retention was ~95% (77%–105%), ~94% (80%–105%), and 39% (31%–50%) after 24, 72, and 120 hours, respectively (Table 2).

3.3. Carotenoids Stability During Porridge Preparation (Wet Cooking)

Carotenoid stability during subsequent wet cooking of fermented and unfermented flours was found to be dependent both on carotenoid structure ($p < 0.0001$) and maize genotype ($p < 0.0001$; Table 3). Total carotenoid content retention after wet cooking of the resulting flours (porridge preparation) ranged from 69% to 95% among genotypes, with average retention through the processing of 80% (Supplementary Table S2). However, no interaction between these factors was observed ($p = 0.555$). In general, carotenoid retention during wet cooking was found to be directly proportional to the number of hydroxyl groups in the structure with the highest retention for isomers with two hydroxyl groups (LUT and ZEA, ~82%); followed by isomers with one hydroxyl group (BCRYP, 66%), and finally, without hydroxyl group (cis-carotenes, 45%; all trans-BC, 30%) among genotypes (Figure 1).

Table 3. Carotenoid contents (µg/g DW) of non-fermented and fermented products after cooking.

Fermentation (hours)	Genotype [1,2]				
	1	**2**	**3**	**4**	**5**
all-trans-lutein					
0	10.9 ± 0.4a	8.0 ± 0.2a	9.4 ± 0.2a	6.4 ± 0.1a	8.2 ± 0.4a
24	10.5 ± 1.2a	6.1 ± 0.5b	4.8 ± 0.6b	4.1 ± 0.3b	6.7 ± 0.8a
72	12.2 ± 1.6a	6.3 ± 0.2b	5.6 ± 0.1b	5.1 ± 0.3b	8.1 ± 0.2a
120	10.0 ± 0.3a	6.6 ± 0.1b	5.6 ± 0.2b	4.4 ± 0.2b	8.0 ± 0.6a
all-trans-zeaxanthin					
0	2.6 ± 0.0a	29.5 ± 0.9a	34.5 ± 0.9a	19.6 ± 0.2ab	19.7 ± 0.8a
24	2.4 ± 0.3a	29.8 ± 2.4a	32.6 ± 0.6ab	25.4 ± 2.4a	22.4 ± 2.0a
72	2.9 ± 0.4a	27.3 ± 1a	31.1 ± 1.8ab	22.5 ± 0.8ab	22.8 ± 1.0a
120	2.3 ± 0.1a	27.4 ± 0.1a	28.4 ± 0.5b	18.6 ± 0.2b	21.1 ± 1.3a
β-cryptoxanthin					
0	0.9 ± 0.0a	1.6 ± 0.1a	2.3 ± 0.1a	1.4 ± 0.1a	1.6 ± 0.1a
24	0.7 ± 0.1a	5.1 ± 1.5a	0.7 ± 0.0b	0.9 ± 0.3a	1.5 ± 0.9a
72	0.9 ± 0.1a	5.5 ± 0.2a	0.9 ± 0.1b	1.3 ± 0.4a	2.9 ± 0.7a
120	0.7 ± 0.0a	4.4 ± 1.1a	0.8 ± 0.0b	1.1 ± 0.3a	2.8 ± 0.6a
all-trans-β-carotene					
0	3.5 ± 0.1a	4.3 ± 0.2a	2.0 ± 0.1a	1.2 ± 0.0a	1.5 ± 0.0a
24	4.9 ± 0.5a	3.0 ± 0.3b	1.3 ± 0.1b	1.0 ± 0.0b	1.6 ± 0.2a
72	4.4 ± 0.9a	2.9 ± 0.1b	1.7 ± 0.1b	1.2 ± 0.1a	1.8 ± 0.0a
120	4.5 ± 0.1a	3.1 ± 0.2b	1.5 ± 0.1b	1.0 ± 0.0ab	1.7 ± 0.0a
total carotenoid content					
0	20.8 ± 0.6a	47.6 ± 1.4a	50.8 ± 1.0a	30.3 ± 0.2ab	33.2 ± 1.3a
24	21.1 ± 2.3a	46.9 ± 4.7a	41.5 ± 1.1b	32.5 ± 2.2a	33.6 ± 3.8a
72	23.6 ± 2.6a	45.6 ± 1.6a	41.2 ± 1.9b	31.6 ± 1.5ab	37.2 ± 1.6a
120	20.4 ± 0.6a	45.1 ± 1.3a	38.2 ± 0.7b	26.4 ± 0.6b	35.4 ± 2.5a
Xanthophylls					
0	14.4 ± 0.4a	39.1 ± 1.1a	46.2 ± 0.9a	27.4 ± 0.0ab	29.5 ± 1.2a
24	13.6 ± 1.5a	41.0 ± 4.3a	38.2 ± 1.0b	30.4 ± 2.1a	30.5 ± 3.4a
72	16.0 ± 2.1a	39.1 ± 1.3a	37.5 ± 1.7b	29.0 ± 1.4ab	33.8 ± 1.6a
120	13.0 ± 0.5a	38.3 ± 1.2a	34.9 ± 0.8b	24.1 ± 0.6b	31.8 ± 2.5a

Table 3. *Cont.*

Fermentation (hours)	Genotype [1,2]				
	1	**2**	**3**	**4**	**5**
provitamin A carotenoids[3]					
0	5.4 ± 0.1a	7.2 ± 0.3a	4.4 ± 0.1a	2.7 ± 0.0a	3.4 ± 0.1a
24	6.6 ± 0.7a	7.0 ± 1.1a	2.7 ± 0.3b	2.0 ± 0.2a	3.1 ± 0.7a
72	6.5 ± 0.9a	7.5 ± 0.3a	3.1 ± 0.2b	2.6 ± 0.3a	4.1 ± 0.4a
120	6.3 ± 0.2a	7.1 ± 0.6a	2.8 ± 0.1b	2.2 ± 0.2a	4.0 ± 0.3a
sum of cis-β-carotene					
0	2.9 ± 0.1a	4.1 ± 0.1a	2.7 ± 0.2a	1.7 ± 0.2a	2.3 ± 0.1a
24	2.6 ± 0.3a	3.0 ± 0.3b	2.1 ± 0.5a	1.2 ± 0.1a	1.6 ± 0.3a
72	3.2 ± 0.6a	3.6 ± 0.2a	2.0 ± 0.1a	1.5 ± 0.1a	1.7 ± 0.1a
120	2.9 ± 0.1a	3.6 ± 0.1a	1.8 ± 0.0a	1.3 ± 0.0a	1.9 ± 0.0a

[1] Columns not connected by the same letter within each carotenoid species are significantly different according to Tukey's HSD test ($p < 0.05$). [2] All data are expressed as μg of carotenoids per g of maize food (dry weight). Carotenoid data are expressed as mean ± SEM; $n = 4$. [3] Provitamin A content in content in β-carotene equivalents = all-trans-β-carotene + $\frac{1}{2}$ (α-carotene + β-crytoxanthin + 15-cis-β-carotene + 13-cis-β-carotene + 9-cis-β-carotene).

3.4. Rheological Characterization of Porridges by RVA

Pasting properties of native and fermented biofortified whole-maize flours are summarized in Table 4. The pasting properties, including PV, HPV, CPV, and BS, significantly increased with the extent of the steeping period. They were higher in all fermented Ogi porridges than in native starch porridges with significant differences ($p < 0.05$) observed among the fermented porridges for the different steeping periods. Hot paste viscosity and cold paste viscosity significantly increased ($p < 0.05$), with a maximum value at 72 h steeping period, among genotypes (Table 4), except the 2013 Orange ISO genotype for which a maximum value was reached at 120 h with significant difference ($p < 0.05$). Spontaneous fermentation on whole kernels from a yellow dent endosperm genotype (C17 × DE3) resulted in an increase in peak viscosity from 81 ± 3.6 cp ($p < 0.05$) to 454 ± 14 cp, resulting in a higher breakdown (170 ± 8.2 cp) and lower setback (53 ± 7.9 cp; Table 4). Peak viscosity for orange flint endosperm genotypes (Genotype 5, Hi27 × CML328 and Orange ISO genotypes) were 223 ± 16.5, 282 ± 20.5 cp, and 409 ± 73 cp, respectively, at 120 h spontaneous fermentation following the same trend that yellow dent endosperm C17 × DE3 genotype showed, but with statistically lower peak viscosity compared with the yellow dent genotype, resulting in a statistically lower breakdown and higher setback ($p < 0.05$). Peak viscosity on fermented flour from Orange ISO 2015 (336 ± 28.3 cp) was lower ($p < 0.001$) than Orange ISO 2013 (482 ± 20.5 cp) at 120 h of spontaneous fermentation, indicating a potential seasonal effect (both genotypes are recurrent selection of the same open-pollinated genotype but from different growing seasons). In general, yellow dent endosperm C17 × DE3 genotype showed higher HPV and CPV values compared with orange flint endosperm.

Table 4. Effect of steeping on the final viscosity of the biofortified maize flours from hot flour in water dispersion using a rapid visco-analyzer (RVA) model RVA-4 series.

Genotype[1,2]	Fermentation (hours)	Peak Viscosity (cP)	Pasting Temperature (°C)	Hot Paste Viscosity (cP)	Cool Paste Viscosity (cP)	Breakdown (cP)	Setback (cP)
1	0	81 ± 3.6c	74 ± 2.3a	77 ± 2.9c	175 ± 5.8c	4 ± 1.0c	94 ± 5.3ab
	24	211 ± 19.1b	76 ± 0.2a	187 ± 11.8b	328 ± 31.1b	24 ± 7.5c	117 ± 12.1a
	72	412 ± 9.8a	74 ± 0.5a	297 ± 9.0a	545 ± 16.2a	116 ± 3.2b	133 ± 7.2a
	120	454 ± 14a	74 ± 0.5a	284 ± 11.1a	507 ± 17.7a	170 ± 8.2a	53 ± 7.9b
2	0	73 ± 2.1b	80 ± 2.4a	95 ± 1.1b	151 ± 1.6b	−23 ± 1.5b	79 ± 3.5c
	24	70 ± 3.0b	76 ± 0.9a	108 ± 5.9b	144 ± 8.3b	−38 ± 3.1b	74 ± 5.6c
	72	229 ± 9.1a	77 ± 0.3a	265 ± 4.0a	441 ± 7.9a	−36 ± 9.3b	212 ± 11.9a
	120	282 ± 17.5a	76 ± 0.7a	242 ± 21.5a	426 ± 39.8a	40 ± 4.5a	144 ± 22.5b
3	0	73 ± 2.0c	78 ± 0.9a	89 ± 4.1c	167 ± 6.0c	−16 ± 2.2c	94 ± 5.2b
	24	75 ± 4.0c	78 ± 2.3a	88 ± 10.7c	139 ± 13c	−13 ± 11.7c	65 ± 13.7b
	72	202 ± 15.5b	75 ± 0.2a	180 ± 11.6b	331 ± 22.1b	22 ± 4.0b	130 ± 7.3a
	120	482 ± 20.5a	74 ± 1.2a	301 ± 18.2a	554 ± 32.9a	181 ± 8a	72 ± 18.9b
4	0	71 ± 3.3c	80 ± 1.3ab	83 ± 4.8b	165 ± 7.3b	−12 ± 2.6c	94 ± 4.5bc
	24	78 ± 7.9c	80 ± 0.6a	92 ± 8.2b	141 ± 15.6b	−14 ± 2.9c	63 ± 8.9c
	72	258 ± 10.3b	76 ± 0.8ab	237 ± 7.7a	467 ± 10.1a	20 ± 3.7b	210 ± 3.9a
	120	336 ± 28.3a	74 ± 0.8b	252 ± 10.3a	481 ± 17.8a	85 ± 18a	145 ± 10.5b
5	0	36 ± 1.8c	75 ± 1.9b	43 ± 0.8b	60 ± 1.6b	−7 ± 2.0b	24 ± 1.7b
	24	35 ± 10.0c	82 ± 2.0a	44 ± 15.0b	63 ± 26.2b	−9 ± 6.3b	29 ± 16.8b
	72	99 ± 13.6b	77 ± 0.5b	130 ± 15.9a	220 ± 30.8a	−32 ± 3.9b	121 ± 17.1a
	120	223 ± 16.5a	77 ± 0.4ab	169 ± 13.0a	303 ± 25.0a	54 ± 5.5a	80 ± 9.5ab

[1] Different letter within columns in each genotype indicates a significant difference according to Tukey's HSD test ($p < 0.05$). Mean ± SEM; $n = 4$. [2] All data are expressed in centipoise units (cP).

4. Discussion

The primary purpose of this study was to evaluate the impact of steeping on carotenoid stability as a marker of the potential delivery of carotenoids through traditional African maize porridges. A secondary objective was to assess the changes in pasting properties on the resulting food product to characterize textural properties related to consumer acceptance of biofortified maize and potential impact on carotenoid stability. Five experimental maize (*Zea mays L.*) genotypes (Table 1) were chosen based on their unique carotenoid profiles and different kernel characteristics. Total carotenoid levels in these genotypes were generally consistent with previous results reported by our group, Ortiz et al. [6].

Ogi, a fermented cereal porridge generated by steeping whole kernels and consumed by nearly 150 million West Africans [36], was selected as a model to test the impact of steeping on carotenoid stability. Significant variability in the procedures used by native African communities to ferment cereals has been reported in the literature. Several authors reported differences in the starting materials [36–38] (guinea corn, millet, sorghum white, and yellow corn), milling methods (whole kernels or degerminated flours), number of fermentation periods involved, number of times that the steeping water is replaced by freshwater, and number of thermal processes involved [28,39–42]. Given this context, it was essential to begin to define fermentation parameters that may best preserve pVACs and allow for optimal textural characteristics aligned with consumer preferences. In general, ogi preparation involves an initial fermentation period called steeping with a duration between 24–120 h. The second fermentation period is souring with durations between 12–48 h. As this is one of the first studies to investigate the impact of fermentation on the stability of carotenoids from biofortified maize genotypes, the decision was made to simplify the process and ferment whole biofortified kernels with only one steeping period (with durations of 0, 24, 72, and 120 h) without changing the steeping water. Expecting this would serve to minimize losses due to leaching and therefore provide a baseline for future investigations. The pH of biofortified Ogi flours obtained in this study was found to have a higher pH (~4.97) than reported previously (3.7–4.06) for conventional Ogi flours after the steeping period [28,37,38,43–45]. Genotypes 3 and 4 tended to produce products with a slightly higher acidity after the steeping period in comparison to the other genotypes. Ogi relies on spontaneous fermentation [46], thus differences in the local lactic acid bacteria species populations in Africa and Indiana, USA may have contributed to different fermentation outcomes. This latter has been previously reported in native maize varieties (white vs. yellow) [47] and may also be responsible, in part, for differences in the outcomes of these experiments compared to other fermentation experiments. In addition, maize kernels used in this experiment were stored for 2-years at −20 °C prior to steeping to induce spontaneous fermentation. Therefore, it is possible that these storage conditions might have altered the native microbiota in a way that could result in an incomplete fermentation.

Carotenoid stability was significantly impacted by the duration of the steeping period ($p < 0.001$) across all biofortified maize genotypes. In general, the overall carotenoid retention was 92%, 82%, and 37% after 24, 72, and 120 hours, respectively. Li and others [26] reported 10% β-carotene (95% CI 8.5–11.9) and 21.5% zeaxanthin (95% CI 14.6–28.4) losses on a wet maize flour that was allowed to spontaneously ferment at room temperature (30 °C) in the dark for 48 h (pH 4). Carotenoid retention during spontaneous fermentation has also been investigated on cassava. Aragón and others [25] reported that fermentation of biofortified cassava roots significantly decreased levels of β-carotene equivalent with retentions ranging from 72% to 96% for β-carotene equivalent, with some cassava genotypes showing superior carotenoids retention during spontaneous fermentation. Thakkar and others [23] found that fermentation of grated cassava for three days at room temperature in the preparation gari resulted in a minimal loss (8% loss) of the total β-carotene content. Carotenoid degradation can be induced by light, oxygen, extremes in pH, or a combination of all three [48]. However, light exposure and pH might not be the main degradative factors, since maize fermentation was carried out at controlled temperature (27 °C) with no light exposure, and in general, carotenoids are reported to be stable to pH changes in foods over the range pH 2–7 [26]. Even though dissolved

oxygen in the steeping water decreases during ogi fermentation [45], direct oxidation might be a factor contributing to the full range of carotenoid losses observed.

Carotenoid losses during fermentation may also be attributed to carotenoid leaching in the discarded liquid during food processing. Bechoff et al. [49] reported significant provitamin A carotenoids losses that were attributed to physical losses of pVACs and chemical losses (oxidation) during the processing of biofortified cassava. Nevertheless, one beneficial effect of fermentation is an improvement in extractability and bioavailability of minerals in cereal matrices [50,51]. This latter is achieved primarily by the reduction of phytic acid, a powerful chelator agent for minerals. This fact might have an indirect role in the oxidation of carotenoids during fermentation, since fermentation may enhance mineral release from the food matrix and directly impact stability through their ability to stimulate oxidative processes and carotenoid oxidation through electron transfer reaction mechanism even at lower oxygen tensions [48]. While not directly assessed in this experiment, it is plausible that the positive effect that fermentation has for mineral availability might also be responsible for a negative effect on carotenoid stability, particularly during subsequent processing.

Wet cooking of maize native and fermented whole flour further reduced total carotenoid content with retention ranging from 69% to 95% among genotypes, with average retention through the processing of 80% (Supplementary Table S2). Kean et al. [52] reported TCC retention of 52% in whole yellow dent cornmeal wet cooked with a 10% vegetable shortening and continuous stirring at 95 °C for 20 min. However, the cooking conditions used by Kean et al. [52] might have enhanced carotenoid extraction into bulk lipid and susceptibility to oxidation over the extended cooking period (20 min). Lipkie et al. [32] working with biofortified sorghum porridges made similarly to the present study reported retention of all-trans-β-carotene of ~77% (48%–100%) after 5 min wet cooking of sorghum slurries in boiling water stirred by hand. Carotenoid stability during wet cooking was found to be dependent on carotenoid structure ($p < 0.0001$), with xanthophylls showing superior stability (82% retention) compared to carotenes (30% retention) during wet cooking (Figure 1). A similar finding was reported in wheat flour [53]. Carotenoids are known for properties as a chain-braking antioxidant in scavenging and quenching singlet oxygen. Mechanistic studies on the radical scavenging properties of carotenoids showed that the ability of carotenoids to scavenge radical cations increase with the extension of the chromophore and maximum overlap of the carbon-carbon double bond molecular orbital [54]. Thus, the presence of additional hydroxyl groups on the carotenoid structure and the decreasing number of coplanar conjugated bonds decrease xanthophyll's reactivities in radical scavenging reaction and, as such, would make them less susceptible to oxidative reactions [55,56], which is consistent with our results (Figure 1).

The pasting properties are reflected as changes in viscosity during heating of a suspension, and the changes in the starch physical and chemical structures [57]. In our study, fermentation increased the viscosity for Ogi porridges, achieving the highest value after 120 h of steeping period among all genotypes evaluated. A similar phenomenon was observed by Osungbaro [40] as well as by Adeyemi and Beckley [37] in the natural fermentation of white maize grains (FARZ-27 and DMR-white varieties). They reported an increased viscosity for Ogi porridges of up to 4 days steeping or souring, after which further fermentation led to substantial viscosity reduction. On the other hand, it was noticed that C17 × DE3 genotype showed higher viscosity, HPV, and CPV values compared with genotype 5, Hi27 × CML328, and orange ISO genotypes. Genotype differences in the pasting properties might be related to differences in starch composition among genotypes. Notably, CI7 × DE3 (named C17 × DE exp by Sowa et al. [58]) is a genotype of temperate origin with a dent type of kernel [59]. All other genotypes are partially or entirely of subtropical origin and are of partial or full flint type of kernel. Relative to dent kernels, flinty kernels have a larger relative area of the vitreous or horny endosperm in proportion to the soft starchy endosperm. The vitreous endosperm is related to the compactness of the starch–protein matrix and amylose/amylopectin ratio [60]. Vitreous endosperm has been associated with higher levels of α-zein protein and amylose levels relative to those of floury endosperm [61]. Viscosity is also an indicator of starch hydration. Starch with more branched amylopectin absorbs

and retains more water and achieve high peak viscosities [60]. Therefore, the higher peak viscosity exhibited by the dent type CI7 × DE3 compared to flint type genotypes might be related to its starch and protein composition. Considering these results, genotype effects related to Ogi thickness is a critical factor related to the acceptability of biofortified orange maize given than African consumers prefer high viscosity porridges [42].

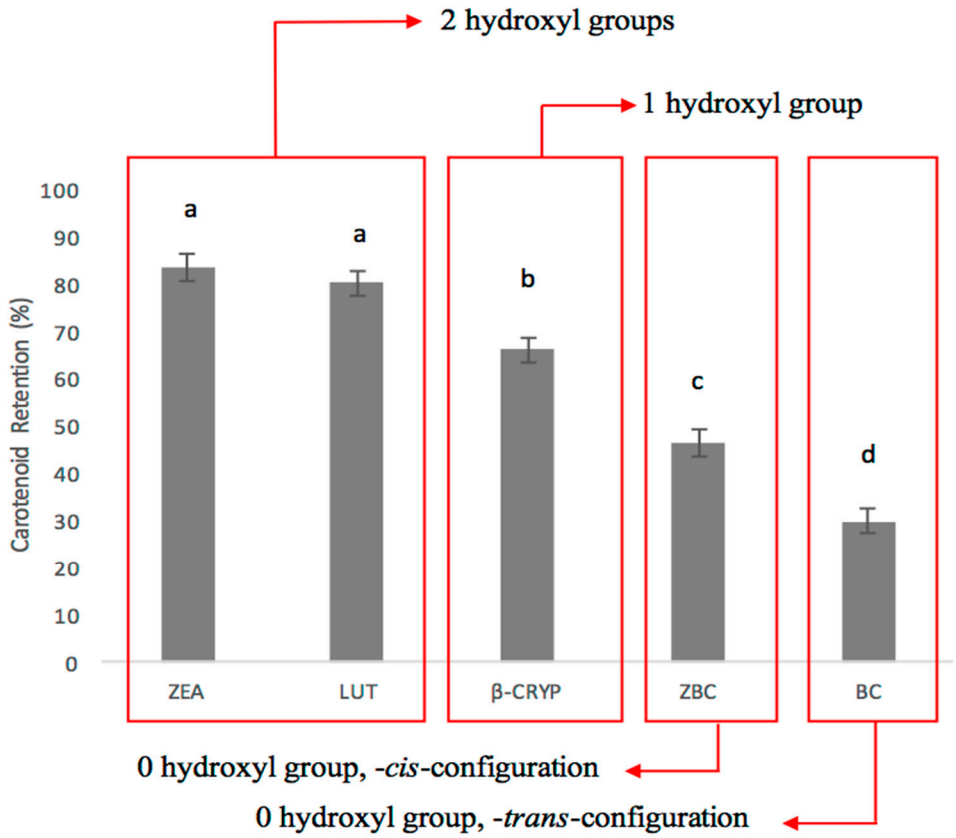

Figure 1. Carotenoids stability during wet cooking of biofortified fermented maize flour. Different letters over the bars indicate significant differences among carotenoids species according to Tukey's HSD test ($p < 0.05$).

Table 4 shows a trend on the setback values among all genotypes evaluated. The setback values of whole maize porridges significantly increase ($p < 0.05$) from 0 h to 72 h steeping time, followed by a significant decrease ($p < 0.05$) from 72 h to 120 h. The setback value in the pasting viscosity curve is likely related to the aging trend and stability. Therefore, the aging trend of fermented whole maize porridge increased significantly with the steeping time compared to non-fermented whole maize flour.

Notwithstanding that natural fermentation of whole maize grain affects the pasting properties of the fermented Ogi by increasing the viscosity and setback with either fermentation or souring time [37,40,42], the effect of natural fermentation on isolated starch from different plant sources was opposite reported [57,62–64]. Natural fermentation on isolated starch decreased peak viscosity and final viscosity on waxy maize, wheat, and cassava starches [62–64]. In addition, the setback of isolated starches decreased with respect to steeping time. A smaller setback value means better stability [65]. The viscosity reduction observed in fermentation of isolated starches could be attributed to the hydrolysis of the starch main molecules, especially those of short-chain amylopectin, associated with both the action of microbial amylases and modest hydrolysis produced by the organic acids generated during fermentation [62]. Structural alteration on the outer layers of the maize bran during spontaneous fermentation of whole maize kernels might have a role on the viscosity increase during the fermentation of maize grains. The outermost structure of maize kernels is a composite plant

material consisting of thick-walled cells originating from the aleurone layer, testa, pericarp, and residual endosperm and is composed mainly of heteroxylans (approximately 67%) and cellulose (22%), but also content significant amounts, phenolic acids (approximately 4%, mainly ferulic and diferulic acid), and acetic acid [66]. Alkali-soluble components of the maize bran also have functional properties as adhesives, thickeners, and stabilizers [67]. As a matter of fact, the release components of the maize bran during alkaline cooking during tortilla preparation improved the viscosity, cohesiveness, and adhesiveness of the masa and tortillas [68]. Therefore, it is possible that the increase in viscosity found as a consequence of the fermentation duration time might be caused by an alteration in the outer layer of the maize grain and/or starch complex formation between starch and components of the maize bran.

In summary, fermentation of biofortified maize grains resulted in a considerable reduction (63%) on total carotenoid content, with a generalized effect across all carotenoids forms after long steeping periods. Natural fermentation might also disrupt starch granules and the release of bran components, which translate into changes in the rheological properties of the end-product. Further studies are need to confirm this hypothesis. The extent to which these effects might enhance the solubility of minerals and soluble fibers, as previously reported, [50,69–71] remains to be fully assessed. However, it appears that these factors would play a role in destabilizing carotenoids during fermentation. Although all carotenoid forms are susceptible to degradative reactions during thermal processing, consistent with previous reports, carotenoid susceptibility was dependent on the number of hydroxyl groups present in the structure with xanthophylls more stable to thermal processing than carotenes. These results suggest that fermented maize products made from steeping of biofortified maize genotypes can serve as a source of pVACs, but compatibility of steeping and thermal processing parameters must be further investigated to ensure that carotenoid stability and, eventually, bioavailability can be maximized in these staple foods.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/9/11/771/s1, Table S1: pH of the slurry water after the steeping period; Table S2: Carotenoid retention during wet cooking (% of starting content).

Author Contributions: Conceptualization, and Methodology, D.O. and M.G.F. Investigation and sample analysis, D.O. and S.G.N. Resources M.G.F. and T.R., Writing—Original Draft Preparation, D.O.; Writing—Review & Editing, M.G.F and T.R.; Supervision, M.G.F.; Project Administration, M.G.F.; Funding Acquisition, M.G.F.

Funding: This research was supported in part by Patterson Chair funds, National Science Foundation (NSF) DBI-0922493 and HarvestPlus (209591) as well as the USAID Feed the Future Food Processing and Post-Harvest Innovation Lab (no. AID- 0AA-L-14-00003). Colciencias and Fulbright-Colombia supported D.O. through doctoral funding.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

pVACs	provitamin A carotenoids
TCC	total carotenoid content
BC	all-trans-β-carotene
AC	α-carotene
BCRYP	β-cryptoxanthin
LUT	all-trans-lutein
ZEA	zeaxanthin
DW	dry weight basis
wb	wet basis
RH	relative humidity
SEM	standard error of the mean
Orange ISO	orange corn in isolated field where it open pollinates

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