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Nonenzymatic β-Carotene Degradation in Provitamin A-Biofortified Crop Plants

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Supporting Information

ABSTRACT: Provitamin A biofortification, the provision of provitamin A carotenoids through agriculture, is regarded as an effective and sustainable intervention to defeat vitamin A deficiency, representing a global health problem. This food-based intervention has been questioned in conjunction with negative outcomes for smokers and asbestos-exposed populations of the CARET and ATBC trials in which very high doses of β-carotene were supplemented. The current notion that β-carotene cleavage products (apocarotenoids) represented the harmful agents is the basis of the here-presented research. We quantitatively analyzed numerous plant food items and concluded that neither the amounts of apocarotenoids nor β-carotene provided by plant tissues, be they conventional or provitamin A-biofortified, pose an increased risk. We also investigated β-carotene degradation pathways over time. This reveals a substantial nonenzymatic proportion of carotene decay and corroborates the quantitative relevance of highly oxidized β-carotene polymers that form in all plant tissues investigated.

KEYWORDS: *vitamin A deficiency, provitamin A, apocarotenoids, biofortification, carotenoid stability, carotenoid cleavage, carotenoid polymer*

INTRODUCTION

Carotenoids are isoprenoid compounds biosynthesized by plants, algae, fungi, and bacteria. In plants, they fulfill essential functions in photosynthesis, as antioxidants[1] and phytohormone precursors.[2,3] When ingested and absorbed, carotenoid species with at least one unsubstituted β-ring gain provitamin A functionality. They undergo oxidative cleavage at the central double bond catalyzed by the enzyme BCMO1 (NinaB in *Drosophila*), delivering two molecules of retinaldehyde (retinal, Figure 1) in the case of β-carotene. Reduction yields all-*trans*-retinol that can be esterified for storage. Provitamin A is the source for two important biologically active compounds. Retinal enters the visual cycle as opsin chromophore. The two-step oxidation of retinol delivers retinoic acid, which is involved in multiple regulatory pathways related to reproduction, cell differentiation, organogenesis, embryonic development, immunity, and metabolic control.[4] Binding to its heterodimeric retinoic acid receptor (RAR)/retinoic x receptor (RXR) receptors, retinoic acid modulates the transcription of target genes.

Vitamin A deficiency (VAD) remains a global health problem. VAD leads to a compromised function of the immune system, exacerbating childhood diseases with frequently fatal consequences. Vitamin A deficiency is also the leading cause of preventable childhood blindness, with up to 500 000 children going blind, annually. Children and pregnant women in low-income populations of Africa and southeast Asia are most affected (WHO; http://www.who.int/nutrition/topics/vad/en/). Supplementation and fortification programs have diminished but not eradicated VAD for reasons of coverage, recurrent costs, and compliance.[5] Providing provitamin A sustainably through traditional agriculture and local trade is an alternative option. The simple fact that "seeds multiply, while pills don't" is the lead idea behind the biofortification concept of HarvestPlus (www.harvestplus.org), pursuing the breeding and release of micronutrient-dense staple crops.

Where sufficient genetic variability for provitamin A content exists such as in maize,[6] sweet potato,[7] and cassava,[8] breeding for this trait is important. However, breeding is not an option when this is not the case such as in rice, when breeding is hardly possible such as with banana or less of an option when varietal recovery is difficult such as with potato and cassava. Genetic modification can fill this gap. For instance, rice,[9,10] banana,[11] cassava,[12] potato,[13] and sorghum[14] have successfully been engineered for increased provitamin A levels.

β-Carotene, a Generally Recognized as Safe (GRAS; FDA) compound, has a long history of safe use as a natural food constituent and as an additive. The conflict with this statement that has stirred debates for decades is in the outcome of the often-cited β-carotene and retinol efficacy (CARET[15]) and α-tocopherol and β-carotene supplements and lung cancer (ATBC[16]) trials conducted in the 1980s. In both of these large scale human intervention trials, very high doses of β-carotene (30 mg/day) were used. In the light of the significant body of evidence that dietary β-carotene and other carotenoids

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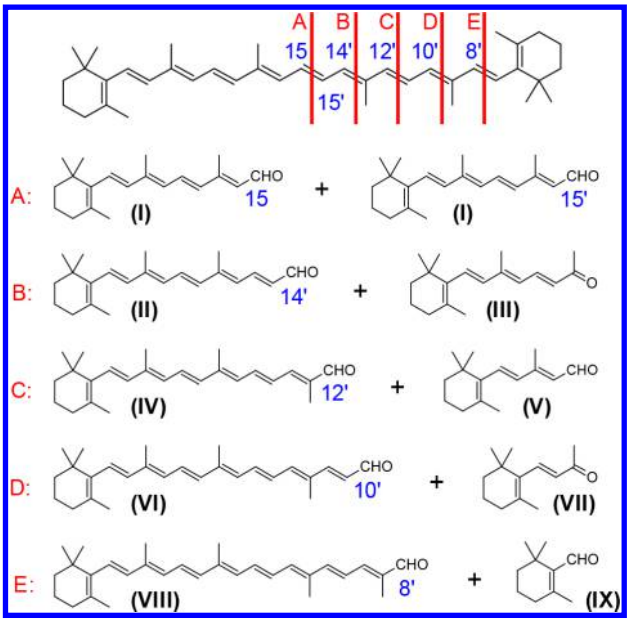

Figure 1. β-Carotene cleavage sites. Cleavage at positions A–E yields the following products: A, retinal (I); B, β-apo-14′-carotenal (II) + β-apo-13-carotenone (III); C, β-apo-12′-carotenal (IV) + β-ionylidene-acetaldehyde (V); D, β-apo-10′-carotenal (VI) + β-ionone (VII); E, β-apo-8′-carotenal (VIII) + β-cyclocitral (IX).

have anticancer activity,[17,18] the unanticipated finding was that supplementation resulted in more rather than less lung cancer among smoking and asbestos-exposed study participants. Adding to the conundrum, other large-scale studies such as the Physicians Health Study involving 11% smokers did not find negative effects upon 12 years of supplementing 50 mg of β-carotene daily.[19,20]

Animal studies conducted in the wake of these trials pointed to the involvement of eccentrically cleaved β-carotene oxidation products, so-called apocarotenoids. Upon high-dose β-carotene supplementation, these exceed normal dietary levels at increased free radical exposure, as experienced upon smoking.[21] Studies with tissue culture cells[22,23] corroborated the potential of apocarotenoids of enhancing the genotoxic effects of oxidative stress. Eroglu et al.[24] reported that certain β-apocarotenoids, namely β-apo-14′-carotenal (II) and β-apo-13-carotenone (III), act as antagonists, reducing the retinoic acid-induced activation of target genes.

Apocarotenoids also form in planta, catalyzed by cleavage dioxygenases (CCDs), a gene family with nine members in *Arabidopsis* (for review, see ref 25). Five of these (referred to as NCED 2, 3, 5, 6, and 9) cleave 9-*cis*-configured xanthophylls, initiating the biosynthesis of the phytohormone abscisic acid and thus cannot form the unsubstituted β-apocarotenoids in question. Additional specialized CCDs are represented by CCD7 and CCD8. The former cleaves specifically 9-*cis*-β-carotene to yield 9-*cis*-β-apo 10′-carotenal, which is converted by CCD8, catalyzing a complex rearrangement plus cleavage to yield carlactone, a precursor of strigolactones.[26] However, CCD8 can also catalyze in vitro a standard single cleavage reaction with the all-*trans* configured apocarotenal precursor to yield β-apo-13-carotenone (III) but with low activity. The determination of the cleavage specificity of remaining CCD1 and CCD4 with (inter alia) β-carotene revealed predominant cleavage sites at positions C9,10, and C9′,10′, yielding β-apo 10′-carotenal (VI) and β-ionone (VII) (CCD1).[27−29]

Besides carotenoid catabolism there is degradation, defined here as the nonenzymatic component of carotenoid decay. Carotenoids are not stable compounds. Due to their polyene structure, they act as antioxidants, a property that is linked to their own destruction, this being fostered by abiotic factors such as temperature, light, metal catalysts, and water content. Oxidizing environments leading to nonenzymatic cleavage can also be initiated by cellular activities. For instance, co-oxidation of carotenoids as a consequence of lipoxygenase activities is long-known. Here, the fatty acylperoxy radicals generated cleave carotenoids by random attack of the polyene chromophore.[30,31] Moreover, the formation of cyclocitral (VIII), β-ionone (VII), and dihydroactinidiolide (a derivative of epoxidated xanthophylls) has been shown to result from photosynthetically generated $^1O_2$.[32] The formation of carotenoid degradation products through reactive oxygen species attack has been investigated in much experimental variation in organic solution (for review, see ref 33). All of the in-chain double bonds present can be attacked, delivering all of the possible unsubstituted apocarotenals/ones of β-carotene shown in Figure 1. Thus, the number of apocarotenoid species formed nonenzymatically surpasses the number of those that are enzymatically formed. This renders retinal and geronic acid as indicators for nonenzymatic decay, since no CCDs with the respective regio-specificity of cleavage is known from plants. Moreover, as outlined above, β-apo-14′-carotenal and β-apo-13-carotenone also represent likely hallmarks for nonenzymatic destruction.

The RAR-antagonizing effect of β-carotene cleavage products discussed led the authors[24] to request the investigation of apocarotenoids in provitamin A-biofortified crops with high levels of β-carotene. We follow this suggestion with the here-presented data. Our analysis and quantification of β-carotene-derived cleavage products across biofortified and nonbiofortified crop plant tissues combined with the calculation of potential exposure document no reason for concern. Moreover, we provide evidence on the different degradation routes that β-carotene takes in complex plant matrixes.

MATERIALS AND METHODS

Materials. *Standards.* Unlabeled apocarotenoids were a gift from BASF (Ludwigshafen, Germany). Triple deuterium-labeled apocarotenoids were synthesized by Buchem (Apeldoorn, Netherlands): D_3-β-ionylidene-acetaldehyde ($C_{15}H_{19}D_3O$) $[M + H]^+$ 222.1932; D_3-β-apo-13-carotenone ($C_{18}H_{23}D_3O$) $[M + H]^+$ 262.2245; D_3-retinal ($C_{20}H_{25}D_3O$) $[M + H]^+$ 288.2401; D_3-β-apo-14′-carotenal ($C_{22}H_{27}D_3O$) $[M + H]^+$ 314.2558; D_3-β-apo-12′-carotenal ($C_{25}H_{31}D_3O$) $[M + H]^+$ 354.2871; D_3-β-apo-10′-carotenal ($C_{27}H_{33}D_3O$) $[M + H]^+$ 380.3027; D_3-β-apo-8′-carotenal ($C_{30}H_{37}D_3O$) $[M + H]^+$ 420.3340. Geronic acid ($C_9H_{16}O_3$) $[M + H]^+$ 173.1172, D_6-geronic acid ($C_9H_{10}D_6O_3$) $[M + H]^+$179.1549, and fully oxidized β-carotene polymer were purchased from Avivagen Inc., (Ottawa, Canada). The polymer was also produced by bubbling air through a stirred solution of β-carotene in ethyl acetate. The dried sample was dissolved in 3 mL of ethyl acetate, and the oxidized β-carotene polymer was precipitated by addition of 50 mL of hexane. After washing with hexane, the precipitate was dissolved in ethyl acetate and precipitated twice.

Biological Materials. Philippine food items were purchased on several markets in the greater Manila area. After lyophilization and determination of the water loss, they were immediately frozen and shipped for analysis. HarvestPlus provided unprocessed field-grown orange maize cobs and yellow cassava storage roots from Zambia; field-grown orange fleshed sweet potato (OFSP) tubers came from Uganda. The identity of the experimental Golden Rice 2 (GR2)

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material in a cv. Kaybonnet genetic background is as detailed elsewhere.[10] Wild-type rice and GR2 were grown in the greenhouse under a 14/10 h day/night cycle at 31/26 °C. Panicles were tagged at flowering date and harvested at 25 ± 2 days after flowering. The seeds were bulked and stored as paddy at ambient temperature. At given times, 5 g aliquots were dehulled, polished, and frozen at −20 °C. At the end of the observation time, seeds were analyzed as described below.

Extraction of Nonvolatile β-Carotene Oxidation Products. Fresh plant tissues with high water content (sweet potato, cassava, fruits, and leafy vegetables) were lyophilized. Determination of water losses allowed relating the (apo)carotenoid content to the original sample. Maize and rice grains were analyzed without further drying. The data thus relate to a raw as purchased fresh weight basis. Plant tissues were ground to a fine powder using a ball mill (MM200, Retsch, Germany). Depending on the pigment content, varying amounts of sample (10−20 mg of green leafy samples and 50−500 mg of nongreen samples) were extracted with 6 mL of acetone spiked with an internal standard mix consisting of 75 pmol of each deuterated β-apocarotenoid and with 50 μg α-tocopheryl-acetate. Following extraction for 2 h at room temperature on an overhead shaker, samples were centrifuged (10 min, 3200*g*), and the cleared supernatants were partitioned against 2 mL of petroleum ether:diethyl ether (2:1, v/v) and 6 mL of water. The epiphase was dried and dissolved in 50 μL of dichloromethane:methanol (1:1, v/v), of which 2 μL were used for LC-MS analysis.

Highly oxidized β-carotene polymers (OxBC) were extracted from OFSPs, carrots, and GR, essentially according to ref 34. Samples (70 g of polished rice seeds, 15 g of sun-dried OFSP, and 15 g of dried carrot flakes) were ground to a fine powder and stirred in 300 mL of ethyl acetate overnight. After filtration and evaporation of the solvent, the residue was dissolved in 2 mL of ethyl acetate, and the polymer fraction was precipitated by dropwise addition of 50 mL of hexane. After centrifugation (10 min, 3200*g*), the pellet was dissolved in 2 mL of ethyl acetate and precipitated twice.

Geronic acid was extracted from 1 g of ground rice endosperm with 2 mL of H_2O and 10 mL of acetonitrile spiked with 25 ng of D_6-geronic acid. Following centrifugation (5 min, 3200*g*), the supernatant was dried and redissolved in 8 mL of $CHCl_3$. After addition of 5 mL of 30 mM KOH, the sample was mixed for 5 min and centrifuged (5 min, 3200*g*), and the aqueous supernatant was recovered. The chloroform phase was re-extracted with another 3 mL of 30 mM KOH. The combined supernatants were adjusted to pH 1−2 with HCl. Geronic acid was partitioned into the organic phase after adding 5 mL of $CHCl_3$. After vigorous mixing and centrifugation, the organic phase was collected, dried, dissolved in 50 μL of MeOH, and subjected to LC-MS analysis.

For the analysis of volatile β-carotene-derived apocarotenoids, the lyophilization step was omitted; this excluded the samples from the Philippines for analysis (see above). Samples were ground to a fine powder in liquid nitrogen and extracted on an overhead shaker for 2 h with 4 mL of acetone spiked with 2 nmol of D_3-β-ionone. After centrifugation (10 min, 3200*g*), the supernatant was partitioned against 0.5 mL of hexane and water. Two microliters of the hexane phase were used for GC-MS analysis. Alternatively, volatiles were analyzed via thermo-desorption using TD-cartridges filled with 200 mg of Tenax TA35/60 sorbent (Markes International Ltd.) for sampling. For solid phase microextraction (SPME) sampling, fibers coated with 100 μm of polydimethylsiloxane were used (Supelco).

Analytical Procedures. Nonvolatile apocarotenoids were analyzed using a LC-MS system (Thermo Scientific) consisting of an UltiMate 3000 UPLC equipped with a photodiode array detector and a Q-Exactive mass spectrometer. Separation was achieved with a Hypersil Gold C18 UPLC-column (150 × 2.1 mm i.d., 1.9 μm, Thermo Scientific) with the solvent system A, 0.1% formic acid in water and B, 0.1% formic acid in acetonitrile using a gradient from 70% B held isocratically for 1 min to 100% B within 4 min at a constant flow rate of 0.5 mL min^{-1}. The final conditions were maintained for 20 min. Apocarotenoids were subjected to atmospheric pressure chemical ionization (APCI) and analyzed in the positive mode. Nitrogen was used as sheath and auxiliary gas, set to 20 and 10 arbitrary units, respectively. Vaporizer temperature was 350 °C, and the capillary temperature was 320 °C. The discharge current was set to 5 μA, and MS^2-spectra were generated by parallel reaction monitoring (PRM) using a normalized collision energy (NCE) of 35 arbitrary units. For quantification, the respective D_3-labeled compounds served as internal standards. Standard curves were obtained with each unlabeled apocarotenoid in a range of 0.5−15 pmol on-column containing a constant amount of 3 pmol of the respective D_3-labeled compound. Biological samples were spiked with all of the labeled compounds, as described above. The TraceFinder 3.2 software (Thermo Fisher Scientific) was used for quantification based on the MS^1 signal, the MS^2 spectra serving as a qualifier. The quantification of β-carotene relied on standard curves covering 8.75 to 280 ng on-column. Peak areas of the photometric signals at 450 nm were integrated; α-tocopheryl-acetate was integrated at 285 nm and used to correct for unspecific losses during sample processing.

Geronic acid was analyzed by LC-MS. A Hypersil Gold C18 UPLC-column (150 × 2.1 mm, 1.9 μm, Thermo Scientific) was used with the solvent system A, 0.1% formic acid in water and B, 0.1% formic acid in MeOH. The gradient was developed from 50% B to 100% B within 10 min at a constant flow rate of 0.25 mL min^{-1}; the final conditions were maintained for 5 min. Geronic acid was subjected to electrospray ionization (ESI) and analyzed in the positive mode. Nitrogen was used as sheath and auxiliary gas, set to 40 and 15 arbitrary units, respectively. The vaporizer temperature was 250 °C, and the capillary temperature was 200 °C. The spray voltage was set to 1.5 kV, and MS^2-spectra were generated by PRM using an NCE of 20 arbitrary units. Geronic acid was quantified using the D_6-labeled compound as internal standard. The standard curve samples covered a range of 0.3−10 ng on-column and contained a constant level of 1 ng of the D_6-labeled compound. The TraceFinder 3.2 software was used for quantification based on the MS^1 signal the MS^2 spectra serving as a qualifier. Volatiles were analyzed by GC-MS, as detailed in the Supporting Information.

Size exclusion chromatography of oxidized polymeric β-carotene was carried out with a Prominence UFLC XR system (Shimadzu) using a Styragel HR2 THF 7.8 × 300 mm column (Waters). The solvent used was tetrahydrofuran at a flow rate of 1 mL min^{-1}. A polystyrene standard (2500 Da, Waters) and monomeric β-carotene (537 Da) served as size markers.

FOX assays were conducted essentially according to ref 35 with adaptations for rice grains, as detailed in the Supporting Information.

For ultracentrifugation, 10 g of fresh OFSP tubers or carrot roots were ground in a mortar. Five grams of GR was ground and soaked in 10 mL of H_2O at 60 °C for 10 min. After centrifugation (5 min, 100*g*, OFSP, carrot; 5 min, 3200*g*, GR), 2 mL of the supernatants were layered onto sucrose step gradients (10, 30, and 40%, w/v, in 67 mM phosphate buffer pH 7.5; 10.5 mM $MgCl_2$) and centrifuged (1 h, 72 000*g*). β-Carotene crystals were recovered from the 10/30% boundary.

RESULTS AND DISCUSSION

Philippine Vegetable and Fruit Basket. Aiming at an Asian context, we analyzed 102 plant food items from the Philippines. Some unpigmented ones were analyzed because of known cases where the rate of catabolism exceeds the rate of carotenoid biosynthesis[36−38] so that carotenoids do not accumulate while apocarotenoids might. Because frequently consumed yellow/orange colored soft drinks often contain β-carotene and β-apo-8′-carotenal formulations, some of these were also analyzed. Furthermore, provitamin A-biofortified crop plants were examined.

β-Carotene-derived apocarotenoids eluted in the order of their chain lengths (Figure 1S) and were identified by retention time, exact mass filters, and MS^2 spectral characteristics. Quantification was carried out using internal standardization with the respective triple deuterated compounds. Apocarote-

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noids shorter than C_{15}, i.e. β-cyclocitral (IX, C_{10}) and β-ionone (VII, C_{13}), could not be assessed quantitatively because of their volatility upon freeze-drying (see Methods section) as well as their indifferent partition behavior upon extraction.

Because of size, the data set is provided online (Table S1); numbers in brackets in the text refer to the entries given there. The data show that all of the possible nonvolatile β-carotene-derived apocarotenoids were detected (Figure 1) without exception in samples with substantial β-carotene levels. This includes retinal, β-apo-14′-carotenal, and β-apo-13-carotenone, of which the first and probably the latter two reflect nonenzymatic formation (see Introduction). All three represent compounds for which biological activity in humans is known (retinal) or has been proposed based on cell culture experiments.[24] Some items with undetectable β-carotene showed low levels of derived apocarotenoids such as egg plants (31 and 32), some cabbages (48), sugar beets (63), and bitter gourd (81), indicating background carotenoid biosynthesis dominated by cleavage. Thus, β-carotene-derived apocarotenoids are very common constituents in all categories listed: nongreen vegetables (NGV), green leafy vegetables (GLV), cereal grains (C), and fruits (F). Similarly, the yellow colored soft drinks (SD) analyzed show the presence of β-carotene (120−124 and 126−128). Consequently, they also contained the whole suite of apocarotenoids. The soft drink (125) may well receive its apocarotenoids from the degradation of longest in the series, β-apo-8′-carotenal, of which all of the shorter chain compounds can be derived. This apocarotenoid is used worldwide as a food colorant (INS no. E 160e; syn. CI Food orange 6); the European Safe Food Authority (EFSA) has concluded an acceptable daily intake (ADI) of 0.05 mg/kg body weight. It represents a potential provitamin being cleaved by BCO1 in vitro.[39]

Retinoic acid that might eventually form from retinal was not identified with certainty in GR and other food items because of very low abundance (ca. 1% of the apocarotenoid content). As by peak areas, well-known apocarotenoid 5,6-epoxides[40] were also present across food items, comprising ca. 10−15% of the apocarotenoids analyzed, but could not be quantified due to lacking the respective deuterated standards.

Categorizing β-Carotene Stability. The ratio of β-carotene and the sum of derived apocarotenoids detected can serve as a measure for β-carotene stability. As shown in Figure 2S, there is no simple correlation across food items. β-Carotene containing plant tissue can be highly degrading such as in sili leaves (79), camote tops (61), and Kangkong (59) or provide a regime of relative stability such as with all OFSP lines tested (e.g., 3 and 6), carrots (50), mustasa (82), and pechay (54). Ordering according to percent apocarotenoids detected (Figure 2) places carrot roots and OFSPs into a most stable <0.6% group, followed by the bulk of NGV and by GLV in the <1.8 and <5.5% groups. A storage time course series (1−215 days after harvest) was conducted with seeds of an experimental Golden Rice (GR) cv. Kaybonnet line (marked with arrows), a Tropical Japonica genotype that was investigated because of its relatively high degradation potential. This shows a migration through all the different categories over storage time, starting at day 1 in the <1.8% group. It is important to note that this is not due to increased apocarotenoid accumulation (they remain fairly constant at ≈400 ng g^{-1} in all samples) but caused by a continuing loss in β-carotene, decreasing from 31 to 2.7 μg g^{-1} after 215 days of storage (19−27). Thus, apocarotenoids formed and β-carotene lost do not correspond quantitatively. It

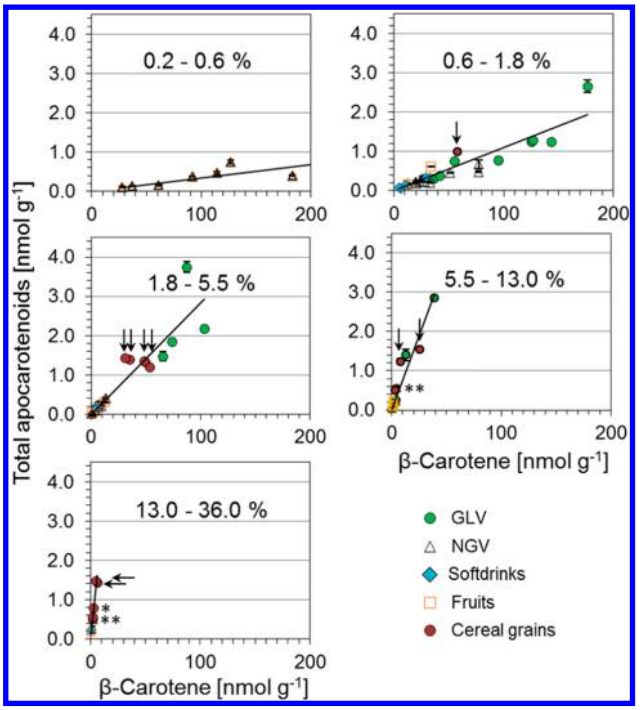

Figure 2. Categorizing β-carotene stability. Ranges of apocarotenoid in mol % show the NGVs as the least degrading background. For further explanations, see text. Asterisks denote orange maize grains (6−10), and arrows indicate the GR storage time course (19−27). The categories are color-coded as given.

is not surprising that orange maize grains of five different lines all group closely together (asterisks in Figure 2) because all were analyzed after three months of storage. Fruits are found scattered across all stability categories except the most stable one; this reflects differences in protective or destructive environments and/or degree of maturity. The soft drinks examined showed a similar distribution.

Two items are not displayed in Figure 2 because they are out of scale with their exceptionally high apocarotenoid content. This refers to taro leaves sold as a dried item (91 and 92) containing 3.1−4.6 μg g^{-1}, about 10 times the amount present in fresh leaves (93 and 94). This relates to the dry mass vs fresh weight comparison; however, there is also an approximate triplication in the apocarotenoid proportion, most likely caused by the processing procedure.

The effect of drying on β-carotene degradation was tested with OFSP (Figure 3S). This shows that the whole suite of apocarotenoids is present already in fresh OFSP. They are increased 1.6- and 1.7-fold by sun drying and oven drying, respectively, with the largest incremental contribution by β-apo-13-carotenone (2.1- and 2.3-fold), β-apo-12′-carotenal (2.4- and 2.1-fold), and β-apo-10′-carotenal (1.4- and 2.1-fold). However, as a general principle, the amount of β-carotene lost is much higher than the sum of apocarotenoids formed, the latter accounting for only 0.3−0.4%.

Across all food items, in ≈70% of all cases, β-apo-13-carotenone was the predominant apocarotenoid, which may be attributed to the fact that it represents the only ketone in the series exhibiting reactivity lower compared to that of aldehydes. This is followed by the shortest product, β-ionylidene-acetaldehyde (24%), while no clear rank order can be attributed to other cleavage products. Evidently, apocarotenoids do not form according to general principles.

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Reasons for Stability: Mode of Cellular Deposition. Carotenoids are mainly protein-bound in chloroplasts, while in chromoplasts, they are sequestered into lipid droplets (plastoglobules), protein−lipid complexes (fibrils), proliferated membranes, and microcrystals, this defining chromoplast types.[41] Of these, crystal formation, as in tomato fruits and carrot roots, appears simplest, not requiring the activation of biosynthesis pathways other than carotenogenesis.[42] Crystals are thought to provide increased β-carotene stability. The fact that OFSP provided a regime of relative β-carotene stability suggests a deposition in crystalline form. Ultracentrifugation carried out with OFSP cell lysates, and carrots show a floating and a sedimenting β-carotene containing band, the latter containing β-carotene microcrystals (Figure 3). In contrast, GR

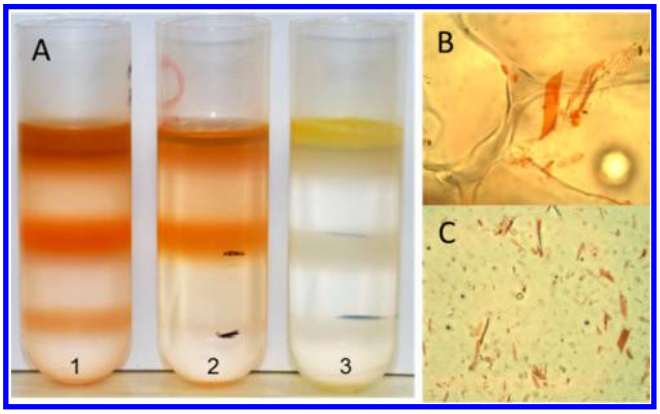

Figure 3. Mode of β-carotene deposition. (A) In sucrose density gradients, β-carotene in OFSP (1), carrots (2), and GR (3) is found in a floating (lipidic) fraction and in a fraction with higher buoyant density, representing microcrystals. (B) OFSP thin section showing β-carotene crystals in situ. (C) OFSP β-carotene microcrystals as isolated from sucrose density gradients.

cv. Kaybonnet rice grains contain β-carotene exclusively in a lipid phase. It is conceivable that the lipidic fraction represents the more bioavailable form of β-carotene. In humans, this results in β-carotene/retinol conversion ratios with GR of ≈3.8:1[43] and ≈13:1 with crystal-containing OFSP.[44]

Quantitative Disproportionalities. It is evident from the OFSP experiment that the amounts of carotenoids lost (a μg g^{-1} range) differ substantially from the apocarotenoids formed (a ng g^{-1} range). The emission of the volatile cleavage products from OFSP chips, namely β-ionone, 5,6-epoxy-β-ionone, dihydroactinidiolide, and β-cyclocitral, has previously been found to be far too low to account for the β-carotene lost.[45]

Very similar inconsistencies also were found with GR paddy in a 215 days storage time course. As Table 1S shows (19−27), β-carotene losses result in sums of apocarotenoids formed that are lower by 2 orders of magnitude. The distribution of β-carotene-derived apocarotenoids (Figure 4) shows that like in OFSP, all possible species are detectable from the beginning. The sum of apocarotenoids increases until ca. days 25−30, during which time ca. 50% of the available β-carotene is degraded. Thereafter, despite continuing β-carotene decay, the sum of apocarotenoids remains constant. Looking at the individual apocarotenoid species, there is little precursor−product relationship discernible, i.e. the longer species that could be precursors of the shorter ones are not favored at early time points, while the shorter ones do not predominate toward the end.

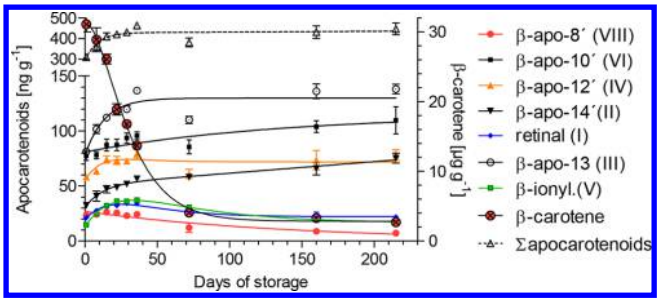

Figure 4. Time course of β-carotene-derived apocarotenoid formation in an experimental (cv. Kaybonnet) GR line. Sampling and analysis were performed as outlined in the Methods section. β-Carotene decays during early phases with a half-life of 25 days, followed by a plateau. Note that β-carotene is given in μg g^{-1} (right axis), while derived apocarotenoids are given in ng g^{-1} (left axis). Roman numerals of cleavage products refer to Figure 1. Data represent the mean ± SEM of three technical replicates.

Apocarotenoids showing a certain downward trend over time are the longest in the series, such as β-apo-8′-carotenal (C_{30}), which indicates some further truncation; however, the same is found with retinal (C_{20}) and the shortest, β-ionylideneacetaldehyde (C_{15}). Any further truncation of the latter would result in volatile species, requiring a different analytical setup (see below). In the end, all curves attain plateaus, similar to an approach to equilibrium. Alternatively, the formation of apocarotenoids might cease with continuing storage in favor of an alternative degradation pathway (see below).

In analogy with the analyses made with OFSP,[45,46] volatile degradation products were analyzed by GC-MS in rice. Significant efforts went into short and long-term sampling of paddy and polished grain because the amounts detected were only minute, not at all reflecting the micrograms of β-carotene lost during storage. An example of a GC-MS trace (Figure 4S) shows the emission of very typical plant β-carotene-derived volatiles, cyclocitral, β-ionone, 5,6-epoxy-β-ionone, and dihydroactinidiolide.

Enzymatic vs Nonenzymatic Degradation. Apocarotenoids can form through CCD-mediated cleavage as well as nonenzymatically. Unprocessed vegetables consist of living cells, so both mechanisms can prevail. However, the general occurrence of retinal (I), β-apo-14′-carotenal (II), and β-apo-13-carotenone (III) across all β-carotene-containing food items reflects a significant nonenzymatic component (see Introduction), even in vegetables and fruits. In contrast, mature cereal grains are dry and the endosperm consists of dead cells[47−49] so that nonenzymatic mechanisms would be expected to predominate. On the other hand, the constant sum of apocarotenoids in older grains (20−27; Figure 4) might suggest enzymatic cleavage to occur at early, liquid-dough ripening stages and cease with cell death. To distinguish, GR was parboiled, a procedure by which paddy is soaked, steamed, dried, and then milled. The heating step[50] would denature proteins, inactivating CCDs. However, parboiling did not attenuate the kinetics of β-carotene decay (Figure 5S). Adding to this, the functional overexpression of *CCD1* in GR as well as its downregulation did not decrease or increase carotenoid levels, respectively.[51] The presence of oxygen should thus remain the single most important factor governing stability. In fact, there is no β-carotene decay when grains are stored in an oxygen-free atmosphere (Figure 6S). In conclusion, the

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J. Agric. Food Chem. 2017, 65, 6588−6598

apocarotenoids detected are predominantly formed non-enzymatically in GR.

Pro-Oxidant Routes and Formation of Polymers. Carotenoids, including β-carotene, are antioxidants, a property that leads to their own oxidation and bleaching. However, they can also foster a pro-oxidant route by successive reactions of carotenoid peroxyradicals with O_2 (for review, see ref 33). Lipid co-oxidation might initiate the process, whereby a lipoxygenase-generated lipid-peroxyradical randomly attacks the polyene chain. In line with this, the RNAi-mediated downregulation of an aleurone lipoxygenase increased β-carotene stability in GR.[52] Lipid peroxidation leads to rancidness and is the cause for the traditional rice milling process, the removal of the lipid-rich aleurone layer from rice grains by polishing.

The ferrous oxidation of xylenol orange (FOX) reaction was employed to probe hydroperoxides in GR endosperm.[53] This revealed that they are more abundant in GR as compared to the WT (Figure 5), indicating pro-oxidant activity of β-carotene.

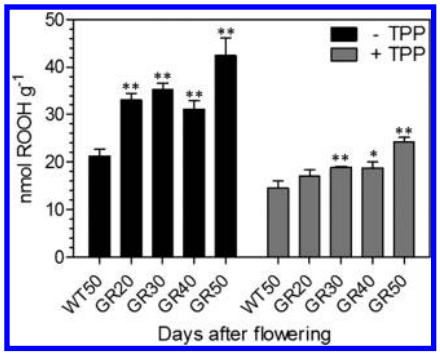

Figure 5. FOX-assays reveal a pro-oxidant degradation route. Hydroperoxides were quantified using the ferrous oxidation of xylenol orange (FOX) reaction before (black bars) and after reduction with triphenylphosphine (TPP, gray bars). WT, untransformed wild-type cv. Kaybonnet rice; GR, Golden Rice. Data represent the mean ± SEM of three technical replicates. *$p < 0.05$; **$p < 0.01$.

FOX assays included measurements before and after TPP reduction to increase the specificity for authentic hydroperoxides. A substantial fraction of 50−60% showed to be not reducible and remained FOX-positive. Like with hydroperoxides, this fraction was more abundant in GR than in the wild type. Nonreducible FOX positive signals have been investigated in great detail in lipid peroxidation[54] and are related to the presence of cyclic peroxides such as monocyclic and serial-cyclic peroxides; however, the extended double bond system in β-carotene also allows cyclic endoperoxide species to form.[55]

Recent publications have highlighted that nonenzymatic oxidation of β-carotene is not dominated by cleavage but instead by the formation of metastable carotenoid-oxygen copolymers (OxBC) that contain 7 to 8 mol equiv of dioxygen that resemble sporopollenins.[56,34] These polymers also form in organic solution to about 85% of the β-carotene used. These compounds have also been reported to prime innate immune functions, providing health-beneficial nonvitamin A functionality.[56] Intriguingly, they also form in significant amounts in plant tissues, especially when dried, independent of the main carotenoid species present.[34]

There is currently no single analytical method capable of assessing OxBCs. They can, however, readily be isolated by hexane precipitation of ethyl acetate extracts. When applied to GR stored for ca. 8 weeks and to dried carrot flakes and OFSP in a comparison, the resulting precipitates showed a comparable size distribution upon GPC and shared similarities in their UV/vis spectra with authentic OxBC (Figure 7S). They lack the long-wavelength absorption characteristics of carotenoids, indicative for engagement of the polyene double bonds in the formation of C−O bonds and/or cross-linking C−O−O-R bonds. Moreover, the formation of OxBC has been shown to be closely correlated with the liberation of geronic acid.[34] This compound can be formally derived from a double cleavage of the β-ring (Figure 6A) and can therefore serve as an indicator of the polymer's β-carotene provenance.

In fact, geronic acid can readily be detected in stored GR samples but is absent from wild-type grains of the same cultivar (Figure 6A). To confirm that geronic acid is a derivative of OxBC, the polymer fraction was isolated from GR and wild-type rice (the latter forming certain amounts of an unspecific precipitate), dissolved in MeOH, and left at room temperature for 8 weeks with a weekly analysis of emitted volatiles, long-chain apocarotenoids, and geronic acid. Remarkably, only the short-chain species β-ionylidene-acetaldehyde (V), β-apo-13-carotenone (III), and retinal (I) were detectable with a steep decrease in abundance with increasing chain length; apocarotenoids > C_{20} were not found. Moreover, the GR-derived precipitate decomposed over time to form certain amounts of volatiles, including β-ionone (trace amounts; not shown), β-cyclocitral, 5,6-epoxy-β-ionone, and dihydroactinidiolide (Figure 6D). Moreover, geronic acid (Figure 6B) was liberated, as expected. These are the same scission products that are found liberated over time from authentic OxBC standards, confirming the findings reported by Burton et al.[56]

Evidently, the early formation of long-chain apocarotenoids (Figure 4) requires the intact polyene for scission that is less available in the polymer so that positions close to the cyclic β-carotene ends remain accessible. The nonenzymatic degradation pathway can thus be interpreted as being two-pronged: initially, cleavage of the intact polyene chain predominates, e.g. by mechanisms related to the well-known Hock scission of polyenic hydroperoxides.[57] This pathway is outcompeted over time by the oxidative cross-linking of β-carotene, forming a copolymer. In support of this, the OxBC formed seemingly accounts for the quantitative discrepancies discussed above. An empirically determined correlation factor suggests that geronic acid detected represents ∼2% of the OxBC polymer present.[34] At day 40 (Figure 4), GR contained 13 μg g^{-1} β-carotene and 0.108 μg g^{-1} geronic acid. After further storage (day 120), 3 μg g^{-1} β-carotene and 0.265 μg g^{-1} geronic acid were detected. This loss of 10 μg g^{-1} β-carotene is compensated by (0.265 − 0.108)50 = 7.85 μg of OxBC polymer, which is quite in the range of the β-carotene loss. The remaining discrepancy may be attributable to the expectedly heteropolymeric nature of the OxBC when formed within complex plant matrixes, possibly involving unsaturated lipid acyl residues. Apocarotenoids do not contribute substantially to OxBC formation, remaining largely unchanged during these late stages of storage (Figure 4).

Geronic acid can also be detected in fresh GLVs, albeit at low levels. Kangkong (59), for instance, contained 50 ng g^{-1} fresh weight. This relates to 350 ng g^{-1} dry mass. Dried gabi taro leaves (92, purchased as dry) revealed 850 ng g^{-1}. This higher level points to increased OxBC formation and/or geronic acid release upon processing. Accepting geronic acid as a valid indicator, this suggests polymer formation across all categories, including GLV.

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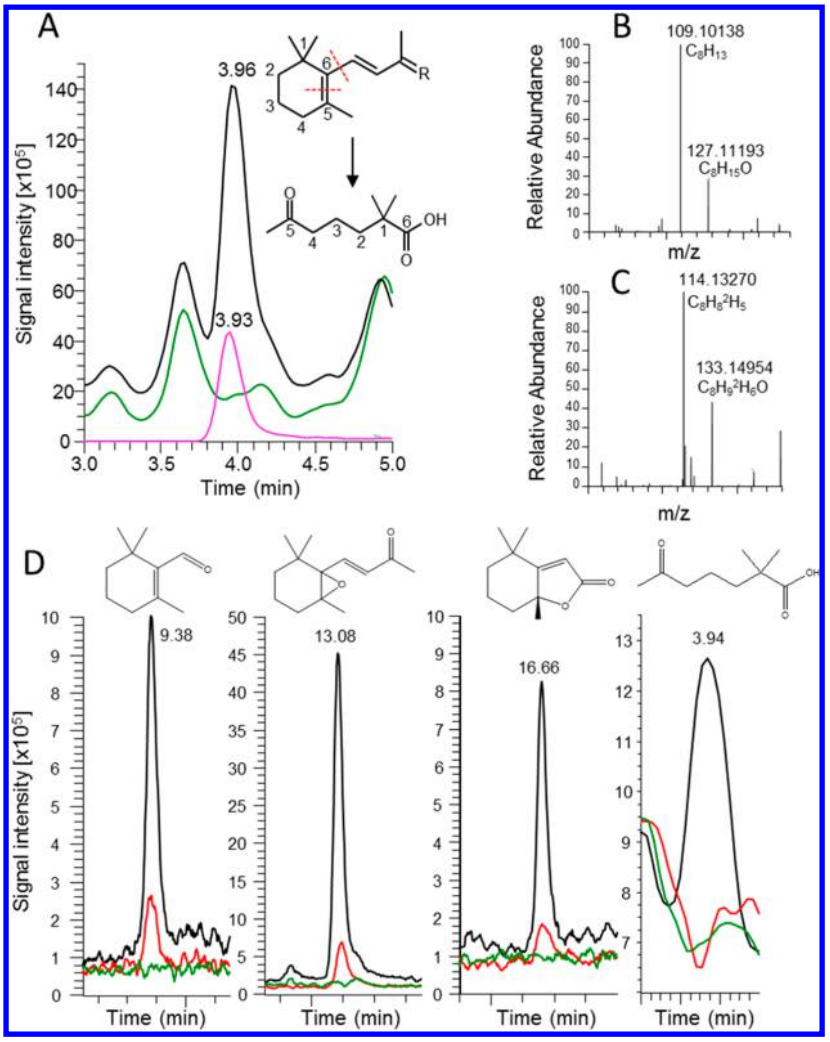

Figure 6. Cleavage products derived from highly oxidized β-carotene polymers. (A) GR and untransformed wild-type rice was subjected to geronic acid extraction and analyzed by LC-MS. Red trace, D_6-geronic acid standard; green trace, wild-type rice; black trace, GR after 90 days of storage. The peak eluting at 3.96 min represents geronic acid detected at its $[M + H]^+$ 173.1172 and verified by comparison of the main MS^2 fragments of the isolated compound (B) and the D_6-labeled standard (C). Alternatively, the polymer was extracted from GR stored for 90 days and kept in MeOH at room temperature (D). The metastable fraction was analyzed after 2 days (red trace) and after 55 days (black trace) by GC-MS, revealing the increasing presence of (from left to right) β-cyclocitral, 5,6-epoxy-β-ionone, and dihydroactinidiolide. The green trace represents the polymer fraction from WT rice. LC-MS analysis (right panel) reveals the liberation of geronic acid from the isolated polymer, detected as in panel A.

Potential Exposure to β-Carotene-Derived Apocarotenoid: A Comparison. The quantitative analyses (Table 1S) allow assessing plant-derived apocarotenoid exposure based on a "raw as purchased" fresh weight basis. For instance, the daily GR (cv. Kaybonnet) intake of 130 g (expressed as weight of dry milled grain) by a 1−3 years old Bangladesh child[58] that was stored for 1 month (23) would lead to an exposure of (431 ng × 130) ≈ ca. 56 μg of total apocarotenoids and of 2184 μg of provitamin A (β-carotene). An estimated 80% cooking retention (Figure 5S) reduces the available provitamin A to 1747 μg. The retinol activity equivalence ratio of 3.8:1 determined in humans[43] would consequently provide 459 μg retinol equivalents (RE); thus, 218% of the estimated average requirement (EAR), which is 210 μg retinol equivalents for this age group.[59] Prolonged storage would not change apocarotenoid exposure significantly but reduce the provitamin A intake (see refs 19−27 and Figure 4).

With the data given (Table 1S), similar calculations on apocarotenoid exposure can be made for all age groups. This can be related to the RE delivered by provitamin A-biofortified staples presented for which retinol activity equivalence ratios have been determined, such as orange maize (10:1; humans[60]), cassava (3.7:1, gerbil model[61]), and OSFP (13:1, humans[44]).

Concerning apocarotenoids with known biological activity in humans, retinal (I) is ubiquitously detected in all β-carotene containing crop tissues, mostly at very low levels ranging from below 10 up to 40 ng g^{-1} (Figure 7A). Higher contents are present in the GLVs (58−62, 78, and 79), the two dried items (92 and 93, not represented in Figure 7) again attaining highest levels of 140 and 280 ng g^{-1}, respectively. The biofortified NGVs, cassava, and OFSP are lowest, mostly in the <10 ng g^{-1} range, and thus not different from other NGVs (black bars). In GR, retinal ranges from ca. 21 to 32 ng g^{-1} during storage, which places it into in a "GLV-like" range. In theory, dietary retinal might contribute to the vitamin A status. Intestinal mucosa enterocytes might convert into retinol by retinal reductase, like the retinal produced through β-carotene cleavage or β-apo-8′-carotenal.[62] However, to our knowledge, there are no reports addressing the fate of dietary retinal.

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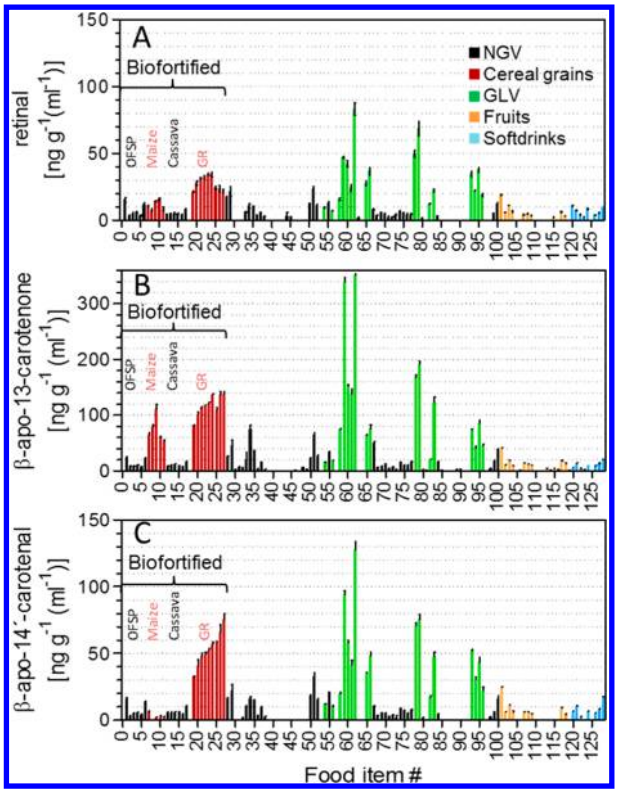

Figure 7. Apocarotenoids quantified across food items. The three apocarotenoids for which biological activity is presumed (as given on the *y*-axes) are displayed. The data are derived from Table S1 in the Supporting Information, and the food item number given refers to this table. NGV, nongreen vegetables; GLV, green leafy vegetables. Soft drink contents are given on a mL^{-1} basis. The two food items purchased as dried products (91 and 92) are not displayed because of their very high contents.

Assuming retinal-retinol equivalency, a woman of reproductive age would need to consume 14.6 kg (dry) GR (23) to cover the EAR of 500 μg retinol activity equivalents (RAE).[59] The retinal content in the soft drinks analyzed is maximally ca. 10 ng mL^{-1} (120 and 128); this would require a daily 50 l consumption to meet the EAR of the same group or, alternatively, the consumption of 27.6 kg of carrots (50 and 51) or of 1.8 kg of dried taro leaves (92). Thus, the potential contribution to the vitamin A status of the available retinal in crops, be they provitamin A-biofortified or not, would be of minor importance.

The β-carotene cleavage products β-apo-13-carotenone and β-apo-14′-carotenal antagonize the retinoic acid receptor in cultured Hep G2 cells.[24] Like retinal, both are ubiquitously detected (Figures 7 B and C) and are present at ng g^{-1} levels in all food items containing substantial β-carotene. β-Apo-13-carotenone is always more abundant than β-apo-14′-carotenal, differing by a factor of 1.5−3.5 in most cases (80%; n = 89).

The biofortified NGVs, cassava, and OFSP are very low in both compounds, in the 10 ng g^{-1} range, while the (stored) cereal grains show higher levels. Depending on storage time, β-apo-13-carotenone is in the 100−120 ng g^{-1} range in GR, while β-apo-14′-carotenal content is 30−80 ng g^{-1}. Maize stored for three months contains 50−110 ng g^{-1} β-apo-13-carotenone but is comparatively lower in β-apo-14′-carotenal. These numbers group provitamin A-biofortified cereal grains together with GLVs (compare red and green bars in Figures 7B and C), so the former can serve as a comparator.

With GR stored for 29 days (23), an EAR of 500 μg RAE (3.8:1, retinol activity equivalence ratio), for example, can be covered by daily consumption of 141 g (Figure 8). Alternatively, relying on white rice, this can be achieved by food diversification. The calculation of the amounts of vegetables needed to attain the same EAR requires the respective vitamin A equivalence ratios in humans, which diverge substantially.[63] For GLV, 10:1,[44] 21:1,[64] 26:1,[65] and 27:1[66] have been reported. Applying the most optimistic (10:1) and an average ratio (19:1) results in the required daily consumption of 145 or 275 g of fresh weight alugbati (Indian spinach), respectively (grid lines in Figure 8). The potential exposure to biologically active apocarotenoids, however, would remain in very comparable ranges; they would, in fact, be ≈16% lower with GR when assuming the 19:1 ratio for alugbati (see Table 2S for numbers). Camote tops, as another GLV example, would require an intake of 171 g when applying the 10:1 conversion, but 324 g would be needed at a 19:1 ratio. In any case, the apocarotenoids in question would be in a very similar range, or up to 4 times higher in the GLV depending on the conversion ratio used. Carrot, for which an retinol activity equivalence ratio of 15:1 has been determined,[64] is often quoted in the context of food diversification for VAD alleviation. Not considering the cultivar (52) because of its low content, the average amount of carrot (50 and 51) to be consumed to attain 500 μg RAE is 160 g fresh weight, thus being in the range of dry GR (141 g). Carrots contain β-carotene in microcrystals and are lower in apocarotenoids, like OFSP (see above). It is therefore not surprising that the apocarotenoid exposure is lower than that in GLVs and GR. Finally, mangoes as an important carotenoid-containing fruit in VAD areas, containing 10.5 μg g^{-1} β-carotene on average (101−103). When the retinol activity equivalence ratio determined for fruits (12:1)[65] is applied, the amount needed is 570 g. This provides the three apocarotenoids in amounts that are very similar compared to those of an intake of 141 g of GR (Table 2S).

In conclusion, our data reveal the ubiquitous occurrence of all possible direct cleavage products and of highly oxidized polymers in plant sources regardless of whether they are biofortified or not. Their formation is mainly driven by nonenzymatic destruction. From a quantitative point of view, no pronounced differences between biofortified and non-biofortified food items can be found upon attaining a given RAE requirement by either biofortified staples or diversified diets.

It has been claimed that provitamin A-biofortified foods bear risks higher than those of conventional crops, attributed to the presence of apocarotenoids antagonizing retinoic acid receptor in tissue culture cells.[24] Linking this finding to the negative outcomes of the CARET and ATBC studies culminated in a frequently echoed press release that "researchers find potential dark side to diets high in β-carotene" (http://researchnews.osu.edu/archive/betacarotene.htm). However, diets have never been accused in this context and, as we show, there is no reason for concern. Apart from the long record of safe consumption in diets, their levels are not out of range, comparatively, in biofortified staples. Supplementation with very high levels of β-carotene (30−50 mg/day) may represent a different scenario because the apocarotenoids in question are not provided in diets but rather form metabolically after

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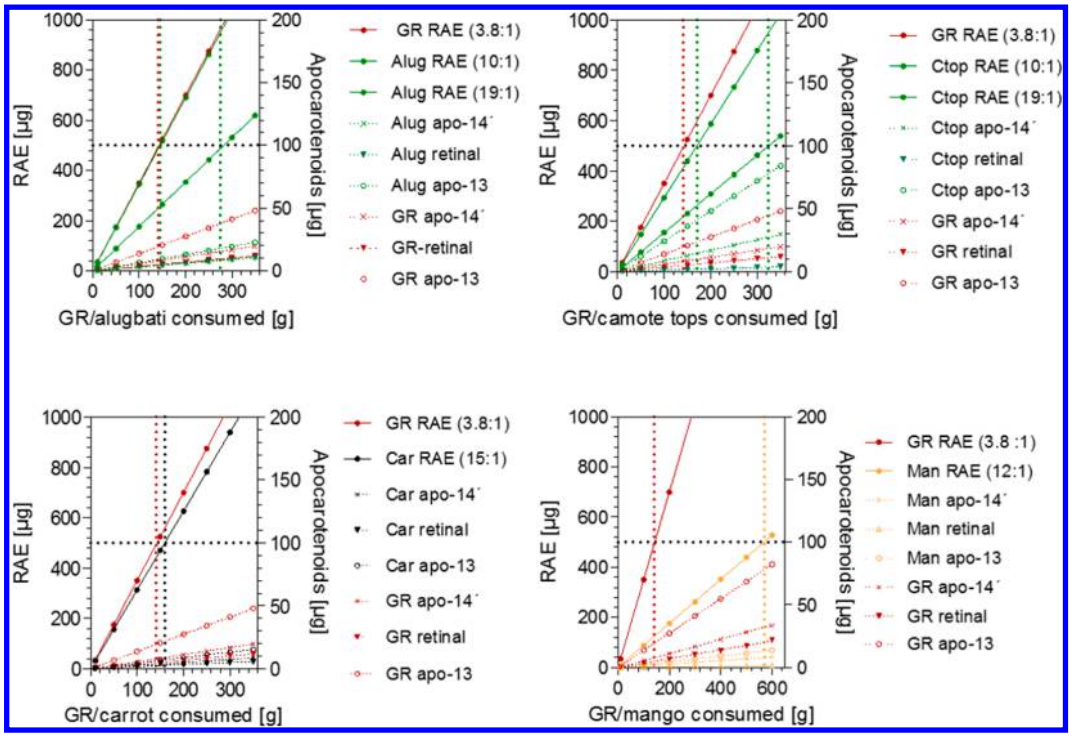

Figure 8. Extrapolation of RAE and apocarotenoid exposure with GR and selected plant food items. The RAE ingested and three apocarotenoids with presumed biological activities, β-apo-14′-carotenal (apo-14′), retinal, and β-apo-13-carotenone (apo-13), are plotted against the amount consumed. The grid line parallel to the *x*-axis denotes the target of 500 μg RAE of an adult Bangladesh woman. The *y*-axis parallel grid lines denote amounts to be consumed to attain the RAE at the given retinol activity equivalence ratio for β-carotene. With the exception of mangoes (consumed fresh), 80% cooking retention of β-carotene was assumed (compare to Table S2). GR: Golden Rice, Alug: alugbati, Ctop: camote tops, Car: carrot, Man: mango.

absorption by people at risk. However, such high β-carotene amounts are not realistically provided by provitamin A-biofortified crops. The highest scoring NGVs are represented by carrots (51) and OFSP (6) containing 68 and 98 μg g^{-1}, respectively, this requiring a daily intake of 441 and 306 g to arrive at 30 mg. Application of RE conversion ratios of 15:1 (carrots) and 13:1 (OFSP) leads to a required effective daily intake of quite unrealistic 3.3 kg of carrots and 2 kg of OFSP.

Supplements normally provide β-carotene in a highly bioavailable form, as in oil, with a retinol activity equivalence ratio that can be low (3.4:1[63]). Unfortunately, the formulations used in the CARET, ATBC, and Physician's Health studies, to our knowledge, have not been defined in this respect. However, the observed yellow skin coloration of trial participants may support the assumption.[67] We thus cannot see reasons for banning the orange carrot (that became provitamin A-biofortified through selection in the 14th century[68]) or any other crop plant high in β-carotene for the avoidance of "dark sides" in populations suffering from deficiency.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.7b01693.

Procedures for volatile analysis and FOX assays, LC-MS separation of apocarotenoids, β-carotene stability plot, effect of drying on apocarotenoid formation, GC-MS analysis of volatile compounds, degradation kinetics upon rice parboiling, carotenoid degradation in oxygen-free environments, GPC analysis of copolymers, quantitative analytical data, and extrapolation of apocarotenoid exposure (PDF)

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Notes

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■ ABBREVIATIONS USED

cv, cultivar; EAR, estimated average requirement; FOX, ferrous oxidation-xylenol orange; GLV, green leafy vegetables; GR, Golden Rice; NGV, nongreen vegetables; OFSP, orange-fleshed sweet potato; OxBC, fully oxidized β-carotene; RAE, retinol activity equivalents; TPP, triphenylphosphine; VAD, vitamin A deficiency; WT, wild type

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