Natural Abundance of 13C in Serum Retinol Differentiates Between Dietary Intakes of C3 versus C4 Plants
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ABSTRACT
Vitamin A is a micronutrient essential in vision, reproduction, immune function, and cellular differentiation. Pro-vitamin A carotenoids are plant sources of vitamin A. The isotopic distribution of 13C and 12C in humans is determined by what foods are consumed. C3 plants, i.e., green vegetables, carrots, and pumpkins, have lower 13C:12C than C4 staple crops, i.e., maize, sorghum, and millet. Vitamin A foods from corn-fed animals will reflect the 13C:12C feed that the animals eat. The serum retinol13C:12C was previously evaluated as a biomarker for vegetable intake. The retinol13C:12C decreased in humans who increased their vegetable intake (range -26.21 to -31.57‰, P = 0.050) and correlated with provitamin A carotenoid intake (P = 0.079). The average δ difference was -0.526 with increased vegetable intake, while control increased by +0.370. A 2X2X2 study in Mongolian gerbils fed white and orange maize or carrots for an extended period of time. Serum retinol13C‰ differentiated between those consuming white maize and white carrots (-27.1±1.2 δ13C‰) from those consuming orange maize and white carrots (-21.6±1.4 δ13C‰, P<0.0001) and white maize and orange carrots (-30.6±0.7 δ13C‰, P<0.0001). This method was applied to Zambian children who had been fed either orange or white maize for two months. Those children who consumed orange maize had a lower δ13C‰ (-26.6±1.98) than their white maize-consuming counterparts (-27.39±1.94) (P = 0.049). In the application of this methodology to efficacy or effectiveness trials, it will be important to choose the appropriate control group and number of subjects for comparison analyses. We investigated changes in serum retinol relative differences of isotope amount ratios of 13C/12C caused by natural 13C fractionation in C3 compared with C4 plants as a biomarker to detect provitamin A efficacy from biofortified (orange) maize and high-carotene carrots.

Keywords: Biofortification; Stable carbon isotope; Vitamin A+; Carrot, GCCIRMS; Vitamin A effectiveness; Vitamin A efficacy

INTRODUCTION
Biofortifying staple and horticultural foods with provitamin carotenoids can sustainably ensure adequate vitamin A (VA) intakes [1], and mitigate potential hypervitaminosis risks caused by preformed VA in high-dose supplements and fortified foods. The bioefficacy of high provitamin A (orange) maize [2], defined as the production of retinol from consumed provitamin A carotenoids [3-5], has been demonstrated in gerbil studies [6,7] and single-meal feeding studies in humans [8,9]. To evaluate health-promoting interventions in humans, efficacy and effectiveness trials are conducted. Efficacy trials are characterized by ideal circumstances that maximize the likelihood of observing a treatment effect: a selected homogeneous population, standardized intervention, and experienced providers or study facilitators. Effectiveness trials are characterized by real-world circumstances designed to determine whether the intervention works as actually used or adopted: a broad heterogeneous population, less standardized intervention protocols, and usual providers [10,11]. A human bioefficacy study determined that orange maize (OM) is an efficacious VA source in children [3], but to our knowledge effectiveness trials have not yet been carried out.

Liver VA concentration is the gold standard for evaluating VA status [12]; however, this is only feasible in animal studies or in special cases in humans. Serum retinol concentrations are homeostatically controlled over a wide range of liver reserves are affected by infection and inflammation [13-16], and are nonsensitive indicators of changes in VA status [12]. Furthermore, several indicators used for VA assessment, such as serum retinol and dose-response tests, are...
qualitative because they only distinguish deficiency from adequacy. Studies performed for provitamin A-biofortified maize in humans have used multiple blood draws for postprandial response [9] or stable isotope methods with intrinsically labeled maize [8] and tracer VA doses for isotope dilution [3]. These techniques require dosing and multiple blood samples per subject, which may not be practical in large-scale effectiveness studies, particularly in children.

Most plants used for food, including staples such as wheat and rice, are C3 plants; however, there are a few notable crops used for human consumption that are C4 plants (e.g., maize, millet, sorghum, sugar cane) [17]. C3 plants discriminate more against 13C during photosynthesis and therefore have lower 13C enrichment than C4 plants [18]. 13C content at natural abundance concentrations is often expressed using the δ notation, which refers to Vienna Pee Dee Belemnite (VPDB) and is expressed as δ13C = [(Rsample/RVPDB) – 1] × 1000. VPDB is relatively enriched compared to most natural materials and has been assigned a δ13C value of 0; therefore, most other natural materials have negative δ13C values. Atmospheric CO2 is relatively stable geographically and topographically and has reported δ13C values ranging from 27.4 to 26.7 [20]. C4 plants typically have δ13C values closer to atmospheric CO2 [e.g., maize [18], sorghum [18], and millet [19–22]]. C3 plants have lower δ13C [e.g., carrots (29.5, 60.2), bananas (226.6, 60.1), and mangos (225.4, 60.1)] [17]. Lipids and other secondary metabolites (including carotenoids) are further reduced in δ13C by 5210 [22,23]; however, the difference between C3 and C4 plants is maintained, as noted with lutein [22] and β-carotene [24]. The δ13C of serum retinol can reflect the dietary sources, including preformed and provitamin A (25). The principle of isotope mass balance states that the amount of heavy isotope in a system is a linear combination of its components [19], which could be used to quantitatively estimate the relative contributions of dietary vitamin A sources.

Several C4 crops are being biofortified with provitamin A carotenoids; in addition to maize, sorghum [26] and millet [27] are also targets. C4 plants often have advantages over C3 plants under conditions of drought, heat, and CO2 or nitrogen limitations, and for this reason they are major crops in tropical and subtropical regions [28]. Furthermore, they may play a vital role in food and nutrition security under changing climates [28,29]. These biofortified varieties should be confirmed for VA bioefficacy and effectiveness at the population level.

This controlled study was undertaken to determine whether β-carotene efficacy from OM could be demonstrated with the use of shifts in the δ13C of serum retinol from the natural enrichment of maize feeding and comparing these values to the δ13C of liver VA, liver VA concentrations, and serum retinol concentrations. The δ13C was determined with GC combustion isotope ratio MS, which is known for its high degree of precision at natural abundance concentrations [30]. Mongolian gerbils are a useful model for human absorption and metabolism of provitamin A carotenoids (31233). Findings in maize could also extend to millet and sorghum because of their similar 13C enrichment.

METHODS

Maize, The bio fortified OM was developed at the International Maize and Wheat Improvement Center in Mexico as part of its Harvest Plus biofortified maize research project [31–34]. Seed was shipped from Mexico to Zambia, and the grain of this OM variety was produced on a commercial farm in Central Province, Zambia.

Orange maize was stored frozen (220°C to 210°C) after harvest. White maize (WM) is a locally consumed variety in Zambia. Both varieties were hand-carried to the University of Wisconsin to be used in this study.

Carrots from the USDA carrot breeding and genetics program were grown by the University of California Desert Research and Extension Station in sandy loam soil in October and harvested in March. Carrots were refrigerated at 2°C until shipped overnight from California to Wisconsin. Upon arrival, they were returned to 2°C until freeze-dried for feed preparation. The genotypes used (i.e., high carotene mass and B2327) were selected for high β-carotene concentrations.

Gerbils were formulated with assistance from Harlan Teklad to meet National Research Council-recommended macro- and micronutrient needs [35]. Foods were 50% maize by weight, [36] and were modified by adding carrots at 1.5% by weight. Maize, carrots, or the VA fortificant provided the sources of VA. The retinyl palmitate used as the fortificant was dry vitamin A palmitate (250,000 IU VA/g; DSM Nutritional Products Ltd.) and was added at a concentration to meet; 50% of estimated utilization rates found in previous studies [2.7 2 5.1 mg retinol activity equivalents (RAEs)/100 g body weight [36,37], resulting in a target concentration of 0.25 mg RAE/g feed. All other feed constituents were constant between groups. Treatment groups were differentiated by 3 factors: OM compared with WM, orange carrots (OCs) compared with white carrots (WCs), and VA fortificant (VA+ compared with VA2) in a 2 3 2 3 factorial design.

Gerbils and study design. Gerbils for this study were a random subset of a larger gerbil feeding study (n = 85). Male 284-d-old Mongolian gerbils (Charles River Laboratories) were group housed during VA depletions (2–3/cage) and treatment (2/cage). Animal handling procedures were approved by the University of Wisconsin College of Agricultural and Life Sciences Animal Care and Use Committee. Gerbils were weighed daily for 2.5 wk and thereafter 3 times/wk. Room temperature and humidity were held constant with a 12-h light/dark cycle. Gerbils consumed ad libitum. During the depletion period (days 1–14), all gerbils consumed WM, WCs, and VA2 feed. After 14 d, a baseline group kill (n = 5) was performed by exsanguination when the gerbils were under isoflurane anesthesia. The remaining gerbils were weight matched and allocated into 8 groups (n = 5–7/group) for the treatment period (days 15–77). After a 62-d treatment period (day 77), a final kill (n = 50) was performed as described previously.

Carotenoid and retinoid analyses, all sample analyses for carotenoids and retinoids were performed under gold fluorescent lights to prevent photo-oxidation and isomerization. Feeds were analyzed for carotenoids by a published procedure for extraction [38] and an HPLC system [36]. Feeds were analyzed for retinol with the same extraction and a minor modification of the HPLC system for retinol [39]; solvent A was acetonitrile:water (92.5:7.5, v/v), and solvent B was acetonitrile:methanol:dichloroethane (80:10:10,
volvol), both with trimethylamine (0.05%, volvol). Serum retinol was extracted with a modified published procedure [40]. Briefly, 1 mL serum, 1 mL ethanol, and 25 mL C23 bapocarotenol in methanol were extracted twice with 1.5-mL hexanes, dried under nitrogen, resuspended in 80 mL methanol, and injected onto the first HPLC system for quantification and primary purification [3]. Liver retinol and retinyl esters were analyzed by a modified published procedure [39]; retinol and retinyl esters were summed first HPLC system for quantification and primary purification [3]. Liver VA (A) and serum retinol (B) concentrations in gerbils were extracted with a modified published procedure [40]. Briefly, 1 g liver and published procedure [39]; retinol and retinyl esters were summed first HPLC system for quantification and primary purification [3]. Liver VA (A) and serum retinol (B) concentrations in gerbils [7]. Liver VA (A) and serum retinol (B) concentrations in gerbils [7].

13°C determinations, Serum retinol was further processed for 13°C content by a published procedure [3], including an additional HPLC purification step, drying under vacuum centrifugation, resuspension into hexanes, and injection onto the GC combustion isotope ratio mass MS system (Figure 1) [25]. A separate aliquot of the liver lipid extract was saponified and extracted [7]; the resulting retinol was purified and analyzed similarly to serum retinol.

Maize and carrot total carbon 13C were determined using an elemental analyzer combined with isotope ratio MS. Retinyl acetate from the VA fortificant was saponified and analyzed similarly to liver VA. All feed samples were analyzed in triplicate. Estimation of maize contribution to dietary vitamin A. The mass balance (isotope balance) equation [19,30] was adapted to the population level and solved for the relative contribution of maize to the total VA intake in terms of serum retinol 13C of the test and control groups (Supplemental Methods):

\[ N_{\text{maize}}/n_{\text{combined}} = \frac{\delta_{\text{combined}} - \delta_{\text{control}}}{\delta_{\text{maize}} - \delta_{\text{control}}} \]

Where, \( n \) is RAEs expressed as moles and the corresponding subscripts “maize” and “combined” refer to contribution from maize and total VA, respectively. Mean serum retinol 13C & is represented by \( \delta \), and the corresponding subscripts refer to treatment groups (control group consuming no OM; maize; group receiving VA only from OM; combined; group consuming VA sources of control group in addition to OM) [38-42]. Experimental groups were fit to this model to examine whether the calculated proportion of VA coming from maize matched analytical data. WMOCVA2, WMWCVVA+, and WMOCA+ were used as 3 control groups; OMOCVA2, OMWCVVA+, and OMOCVA+ were used as the 3 respective test groups that consumed combined sources; and OMWCVVA2 was used as the maize-only group. A bioconversion of 12 mg b-carotene equivalents: mg RAEs was used [12].

Statistical analysis: Values are reported as means 6 SDs. Data were analyzed with the use of SAS version 9.4. Outcomes of interest were evaluated with the use of independent 2-sample, 2-tailed t tests or 3- and 1-factor ANOVA to compare treatment groups and to determine differences between groups with the use of the general linear model procedure as appropriate. Feeds were compared with the use of 1-factor ANOVA. Linear regression was also performed with the general linear model procedure. Post hoc letter groupings between treatment groups were determined with the use of least significant differences. Normality of residuals was tested with the Shapiro-Wilk test; homogeneity of variance was tested with Levene's test. Data failing normality or variance assumptions were analyzed nonparametrically by analyzing ranked data. P < 0.05 was considered significant.

RESULTS

Feed properties. Carotenoid and retinol equivalent concentrations in the feeds had the expected relations. OM provitamin A was predominantly b-carotene (96%) with some b-lycopene (3%) and a-carotene (1%). Carrot provitamin A was mostly b-carotene (65%) but with appreciable a-carotene (34%) and minimal b-lycopene (1%). Maize total carbon 13C was higher than carrots for both OM (211.0 6 0.2 compared with 225.6 6 0.3; \( P < 0.0001 \)) and WM (211.3 6 0.3 compared with 225.3 6 0.1; \( P < 0.0001 \)) varieties; 13C did not differ within carrot or maize varieties (\( P < 0.05 \)). The preformed retinyl palmitate used as the fortificant had a 13C of 227.4 6 1.2, which represents only the retinol portion because the sample was saponified before analysis.

Serum retinol and liver VA concentrations. Serum retinol and liver VA (retinol + saponified retinyl ester) concentrations were plotted (Figure 1: A, B). Liver VA concentrations were highly dependent on dietary VA. The group mean for the treatment group that consumed the least VA (WMWCVVA2) was below the VA deficiency cutoff of 0.1 mmol VA/g liver [14], after treatment. Both provitamin A carotenoid sources increased liver VA concentrations considerably. The mean liver VA concentrations from all groups that consumed OC were not different from each other and were much greater than all groups that consumed WC. Within the WC groups, both groups that consumed OM had higher VA liver concentrations than their respective WM controls. The VA fortificant, which was meant to meet 50% of the estimated requirements of gerbils,

Figure 1: Liver VA (A) and serum retinol (B) concentrations in gerbils after consuming feeds with different combinations of provitamin A carotenoid and preformed VA sources. All values are means 6 SDs (n = 5-7). Liver VA residuals were not normally distributed, and variance was not homogeneous; data were analyzed nonparametrically. Horizontal lines at 0.1 mmol/g (A) and 0.7 mmol/L (B) are the deficiency cutoff concentrations. Labeled means with a common letter differ, \( P < 0.05 \). B, baseline; O, orange; VA, vitamin A; W, white.
did not notably improve VA stores. All groups that consumed the VA fortificant had liver VA concentrations that were similar to their respective controls without the VA fortificant. Nonetheless, the group that consumed appreciable VA from fortificant only (WMWCVA+) had a mean liver VA concentration >0.1 mmol/g and was not significantly different from the baseline group (P = 0.08), which meant they were able to maintain initial VA status. Serum retinol concentrations did not differ between groups despite a wide range of liver VA concentrations, and serum retinol was not correlated to liver VA concentrations (R2 = 0.028). Serum retinol and liver VA did not. Serum retinol and liver VA 013C had good agreement (Figure 2), indicating that the accessible serum retinol pool is highly reflective of the major liver VA store. When serum retinol 013C was analyzed with 3-factor ANOVA, all main effects, the VA 3 carrot interaction, and the maize 3 carrot interaction were highly significant (all P < 0.0001). The VA 3 maize interaction (P = 0.09) and 3-factor interaction were not significant. Because of multiple interactions, 1-factor ANOVA with post hoc analysis was then used to analyze the data, including the baseline group. Serum retinol and liver VA 013C by group showed similar responses to treatment. All groups that consumed OM had significantly greater VA 013C than the corresponding WM controls. By contrast, all groups that consumed OCs had much lower VA 013C values compared with corresponding WC controls. The group that consumed the VA fortificant as the primary VA source (WMWCVA+) had serum 013C that was not significantly different than the VA fortificant (227.9, 60.5 compared with 227.4, 61.2). The group that consumed VA almost entirely (>99.9%) from maize had a serum retinol 013C of 220.6, 6.0.7.

![Figure 2: Serum retinol 013C, plotted against liver VA 013C, for gerbils that consumed diets with varying amounts and sources of provitamin A carotenoids and preformed VA. Only gerbils with values for both outcomes are plotted (n = 40; 3–7/group) along with the best fit line of the data (solid line) and y = x (dashed line). VA, vitamin A.](image)

Estimation of maize contribution to dietary VA: Plots were made with the use of Supplemental Methods Equation 4 of 3 pairs of control and test groups. The proportion of dietary VA predicted from Supplemental Methods Equation 5 in the model agreed well with analytical values; all 3 test group means were within the 95% confidence limits. Supplemental Methods Equation 5 can be simplified to include data from the OMWCVA2 group assuming the group mean adequately represents the serum retinol 013C of gerbils that consumed VA almost entirely from maize (>99.9%). Therefore, the relative contribution of maize to total dietary VA in a population can be estimated by the serum retinol 013C group means of the control and test (combined) groups:

\[ \text{N}_{\text{maize}}/n_{\text{combined}} = (\text{combined} - \text{control})/(\text{control} - 20.6) \]

Experimental and analytical variation: Experimental and analytical CVs were very low for 013C and their corresponding 13C isotopic abundance (13C/total carbon) values. All 013C CVs for individual experimental groups were >3.4% and all 13C isotopic abundance CVs for individual experimental groups were ≤0.23%.

Other studies have applied GC combustion isotope ratio MS to measure per-labeled 13C b-carotene ([53] and lutein ([54]) absorption, enabling the characterization of the appearance and disappearance of carotenoids in plasma after the ingestion of physiological amounts from food.

**DISCUSSION AND CONCLUSION**

The analysis of 013C in serum retinol allowed the determination of provitamin A efficacy from biofortified maize compared with feeds containing minimal VA, provitamin A carotenoids, preformed VA, or a combination of sources when contrasted to an appropriate control group. Furthermore, the relative contribution of maize to total VA intake was quantitatively estimated and verified against analytical determination. The first advantage is high sensitivity to detect provitamin A maize consumption because of low variation within groups that consumed the same feeds. Circulating retinol concentrations are homoeostatically controlled outside of severe hypo- or hypervitaminosis A ([12,42,43] and frequently do not respond to VA interventions [3,14,44,45], both of which were observed in this study. However, they are still often used as a primary outcome to evaluate populations and interventions aimed at improving VA status [46], which may not detect the potential effects of b-carotene or VA interventions that use more sensitive methods [3,44,45]. A second advantage is that a single blood sample after longterm consumption is required. More sensitive methods used as outcomes in VA studies, such as isotope dilution [3,44] or appearance in serum [9], use multiple blood draws. This is undesirable—especially when working with children—and can complicate recruitment and follow-up during studies [47]. Finally, no external isotopically labeled material or VA analogues, which are used for isotope dilution, tracer, and modified dose-response tests [12,14,48], are required. These compounds are often expensive and technically demanding to produce and prepare. Together these advantages show promise for future efficacy or large-scale effectiveness trials to evaluate crop adoption and consumption, especially in populations or settings in which resources are limited and multiple sample collections are not practical.

Variations in natural abundance ratios of stable isotopes (e.g., carbon, nitrogen, sulfur, hydrogen, oxygen) measured from numerous sources (e.g., breath, hair, nails, plasma, RBCs, and specific molecules such as alanine) have been used as biomarkers for dietary origin [49]. An early study noted elevated 013C in breath CO2 after the consumption of sugar [50], and more recent applications have further developed the methodology. For example, 013C was measured from several tissues to assess sugar intake [51], and serum total carbon 013C was lower after an intervention to decrease sugar-sweetened beverage intake [52]. Nitrogen-15 enrichment varies between plant and animal protein sources, and this natural difference has been correlated with meat and fish intake in numerous studies [49]. Reduced serum retinol 013C was demonstrated in response to increased consumption of C3 vegetables containing provitamin A carotenoids, including carrots and pumpkin [25], which we also demonstrated in this study. Theoretically be used to apply our proposed method, although instead of a single CO2 peak with 3 mass traces from the combusted retinol to determine the 13C:12C ratio (Supplemental
), mass distributions of retinol would need to be compared, and adequate precision would first have to be demonstrated. Although enzymatic isotope effects are established in plants and yield differences in 13C enrichments both in classes of metabolites (i.e., starch compared with lipid) within plants [22] and between plants exhibiting different photosynthetic systems [18], it is relatively unknown whether similar effects can be observed for VA in animals and affect organ partitioning given that numerous enzymes participate in VA metabolism [53-55]. Excellent agreement between serum retinol and liver VA δ13C indicates that in a paradigm of constant long-term consumption, the use of serum retinol δ13C is a suitable alternative to represent that in the major liver store.

Serum retinol and liver VA δ13C of treatment groups agreed with data in the literature. Lipids and carotenoids are reduced in 13C by 5–10% compared with total carbon [22,23], which agrees with our results that OC total carbon had a δ13C of 225.6‰, and the treatment group that consumed OCs as the predominant VA source (WMOCV2) had a mean liver VA δ13C of 229.8‰ (a difference of 4.2‰). OM total carbon had a δ13C of approximately 211.0‰, and the treatment group with the only appreciable VA dietary source as OM (OMWCV2A) had a mean liver VA δ13C of 220.5‰ (a difference of 9.5‰). Furthermore, the liver VA δ13C from these 2 groups corresponded well to lutein obtained from marigold (a C3 plant) compared with maize (229.9‰ and 219.8‰, respectively). Serum retinol δ13C was similar in the gerbils that obtained VA from the fortificant only (WMWCV*) to the δ13C of the fortificant itself.

Although natural δ13C enrichment is suitable for distinguishing provitamin A carotenoid efficacy from maize compared with a WM control feed in a trial setting, this study revealed limitations on its use as a purely diagnostic tool for VA status. In addition to the baseline group, 4 treatment groups had similar serum retinol and liver VA δ13C despite widely varying liver VA concentrations. These treatment groups either consumed either both OM and OCs or both WM and WCs, and the resulting enrichment was a mixture of the sources. Although WM and WCs are both very low in provitamin A carotenoids, each contributed small amounts to the feed (maize: 5.2, 61.1 ng β-carotene/g feed; carrots: 4.56, 0.4 Ng β-carotene/g feed), which would be reflected in the δ13C values. However, groups that consumed OM had considerably higher serum retinol δ13C and liver VA concentrations than their respective controls that consumed WM. If a population that consumed OM demonstrated elevated serum retinol δ13C, it could be inferred that their VA status is greater than or equal to a control population that consumed WM depending on the initial VA status of the population. δ13C shifts reflect the consumption of provitamin maize but are not a replacement for evaluating VA status, such as isotope dilution methods [3]. Isotope dilution or dose-response tests could be used on a subset of randomly selected individuals to confirm desired VA status in efficacy or effectiveness studies.

Liver VA concentrations were not different between all groups that consumed OCs regardless of additional VA from OM or the fortificant, likely reflecting the downregulation of provitamin A bioconversion [56,57] and a relatively minor impact of the VA fortificant compared with OCs. Despite this, the serum retinol δ13C was still able to distinguish the feeds in which provitamin A carotenoids were obtained from OM or OCs. This is important considering some populations targeted for biofortification have substantial intakes of VA, even if intake varies seasonally [58,59]. Although these reports highlight a need for more sensitive markers of VA status to ensure interventions do not lead to the chronic overconsumption of VA [2,3], biofortification of staple foods with provitamin A carotenoids can mitigate seasonal gaps in provitamin A consumption and reduce the chances of excessive preformed vitamin A intake caused by the regulation of absorption and bioconversion of provitamin A carotenoids [56,57]. A 62-d treatment period was sufficient to have serum retinol δ13C reflect that in the major body pool (i.e. liver) in this study, but this time requirement in humans likely depends on a number of factors, including the baseline body pool of VA, dietary VA intake, and the rate of VA metabolism. Labeled VA doses mix with body stores within 26 d after administration to adults [60] and 12 d in children [61-68]; however, it will likely take longer for serum retinol δ13C to accurately reflect regular dietary consumption. If the VA pool size increases as the intervention intends, this equilibration time would be shorter.

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