

Title: Factors influencing micronutrient bioavailability in biofortified crops

Short title: Biofortified crops: micronutrient bioavailability

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Factors influencing micronutrient (*i.e.* provitamin A carotenoid (pVAC), iron and zinc) bioavailability in biofortified crops were examined: dietary and human factors are the major factors. Dietary factors are related to the food matrix structure and composition. Processing can improve pVAC bioavailability by disrupting the food matrix but this could also result in carotenoid losses. By degrading antinutrients such as phytate, processing can also enhance mineral bioavailability. *In vivo* interventions show that biofortified crops were overall efficacious to reduce micronutrient deficiency with bioconversion factors varying between 2.3:1 and 10.4:1 for trans- β -carotene and amounts of iron and zinc absorbed between 0.7 and 1.1 and 1.1 and 2.1 mg/day, respectively. Micronutrient bioavailability was dependent on the crop type, presence of fat for pVACs, and of antinutrients for minerals. Human factors relate to micronutrient status that can be affected by inflammation and disease. Understanding the interaction between micronutrients is also essential *i.e.* synergic effect of iron and pVACs; or competitive effect of iron and zinc. Further efficacy trials should consider human status and genetic polymorphism linked to inter-individual variations.

Key-words: carotenoid bioavailability; mineral absorption; biofortified crops; processing, food matrix; genetic factors

Introduction

Biofortification of staple crops is a strategy to help combat vitamin A deficiency (VAD), iron (ID) and zinc deficiencies (ZD) by providing micronutrients to malnourished rural populations in developing countries. According to the World Health Organisation (2015)¹: “Biofortification is the process by which the nutritional quality of food crops is improved through biological means such as conventional plant breeding”. Biofortification may therefore present a way to reach micronutrient-deficient populations whose diet is mainly composed of plant-foods and where supplementation and conventional fortification activities may be difficult to implement and/or limited. Conventional plant breeding or transgenic approaches have been developed in the last decade with biofortified staples crops such as maize, rice, cassava, sweet potato, beans, pearl millet, and wheat, which are currently consumed in developing countries in Africa, Asia, and Latin America.²

Although biofortification can increase provitamin A, iron or zinc content in a staple food, measuring the bioavailability of these micronutrients *i.e.* the absorbed fraction utilised by the human body to improve micronutrients status, yet remains a challenge. High micronutrient content in the food does not guarantee good body absorption; a number of *in vitro* and *in vivo* studies have been conducted to understand the factors influencing micronutrient bioavailability in crops. The factors affecting the carotenoid bioavailability in particular have been extensively discussed in the literature³ and can be summarised in the mnemonic SLAMENGIH proposed by West and Castenmiller.⁴ Amongst them, the dietary factors (Matrix in which the carotenoid is incorporated (M) and Effectors of absorption and bioconversion (E)) and the Host related factors (H) including Nutrient status of the host (N) and Genetic factors (G) seem the major factors affecting not only carotenoid but also mineral bioavailability.^{5,6}

There are only a few human studies on micronutrient bioavailability in biofortified staple crops currently available. This is explained in part by the recent development of biofortified food programs that are still in early stages of growth² and in part by the cost of such studies. However bioefficacy studies using human populations provide the most applicable results because they include host-related factors (*i.e.* diseases, micronutrient status, genetic inter-individual variations).⁷ In earlier studies, it was a challenge to study the conversion of β -carotene (BC) to vitamin A (VA) from vegetable sources because of the lack of accurate methods. However, in the past decades, the development of stable isotope methodology using labelled plant food has facilitated the measurement of the bioconversion of provitamin A carotenoids.⁶ As a result, human studies *i.e.* including biofortified crops, are now becoming easier to implement in the field.

This article focuses on the two major factors - dietary and human factors - that influence provitamin A carotenoid, iron and zinc bioavailability. Determination of the effect of processing on the micronutrients is also important since it is linked to the bioavailability of these micronutrients. Dietary factors can be determined using *in vitro* (to measure bioaccessibility of micronutrients and uptake by intestinal cell lines) and *in vivo* studies, whilst human factors can only be determined in *in vivo* studies. Therefore both *in vitro* and human studies are reported in our review, firstly to help understand the relationship with the food matrix and processing/formulation and secondly to understand micronutrient absorption taking into account human status and inter-individual variation genetic factors.

Effect of processing on micronutrient retention and on the food matrix

Provitamin A carotenoids

Vitamin A (VA) in the diet can be found either as the preformed-vitamin in food of animal origin, or in plants as precursors of VA, called provitamin A carotenoids (pVACs).⁸ Preformed vitamin A from meat is more bioavailable than carotenoids from plants. However, most people at risk of micronutrient deficiencies in developing countries have a diet mostly composed of plant foods.

The differences in carotenoid location and form strongly affect their intestinal absorption and therefore their bioavailability.⁹ In fruit and vegetable cells, such as those of biofortified staple crops, carotenoids are embedded in organelles called chromoplasts that have different storage structures (globular or crystalline type).¹⁰ The type of chromoplast is also linked to the type of crop and the crop may therefore have a major impact on the bioaccessibility from the food matrix. In addition bioaccessibility is influenced by the interaction with other dietary compounds such as lipids, fibres, phytosterols and other carotenoids during digestion and absorption.¹¹

Other factors that also influence the food matrix and hence bioavailability, are particle size (*e.g.* puree *vs.* chopped *vs.* intact; food *vs.* supplements) and processing (*e.g.* raw *vs.* boiled or. dried *vs.* fried food).¹¹

Sweet potato

In orange fleshed sweet potato (OFSP) the processes ranked in decreasing order of pVAC retention were: boiling/steaming (80-90%)¹²⁻¹⁴, roasting, frying (70-80%)¹⁵ and sun/solar drying (60-80%)^{12, 15-19} (Table1).

Sun-drying was considered the most damaging process. However sun/solar/-drying was able to retain a major amount of the carotenoids if carried out in a limited time and under good drying conditions (low humidity and high temperature) and this was demonstrated in Uganda¹⁷ and Mozambique¹⁹ (85-90%). In fact the main cause of pVAC degradation was the storage of dried sweet potato following the drying process.^{17,18,20,21} The presence of oxygen and temperature, which both chemically oxidise the trans- β -carotene through free radical mechanisms, were the prime causes.²¹ The retention of carotenoids in dried orange fleshed sweet potato stored at ambient temperature was 20-30% after 4 month under field conditions in Uganda and Mozambique.^{17,20} and this was in agreement with the model prediction.²¹ Retention was also varietal dependent.^{17,22} Bechoff *et al.*¹⁷ showed that varieties with higher dry matter had higher retention of carotenoids. High carotenoid content tended to be inversely correlated to the dry matter content due to a genetic link between dry matter content and carotenoid concentration; therefore varieties with high carotenoid content tended to have lower carotenoid retention.

Cassava

Provitamin A carotenoid retention for yellow cassava (YC) under different processing conditions is in some ways a similar story to OFSP. Overall, boiling retained the most provitamin A (90%)²³ and extensive processing such as the making of gari (a semolina-like fermented and roasted food product) resulted in less than 50% retention^{23,24}. Retention was also

variety dependent: retentions of beta-carotene in seven yellow sweet cassava varieties after boiling were variable (32-94 %).²⁵ During storage, a significant loss in pVAC in flour²⁶ and gari²⁷ made out of yellow biofortified cassava was reported. Both studies showed that at ambient temperature, the degradation of carotenoids was quite dramatic: in Bechoff *et al.*²⁷ gari with an initial *trans*- β -carotene content of 11 $\mu\text{g}\cdot\text{g}^{-1}$ lost 60% of BC after only 2 months.. Biofortified sweet potato and cassava had different initial pVAC contents: whilst OFSP raw roots had levels of *trans*- β -carotene that could reach 300 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight basis.¹⁶, maximum levels in YC were ten times less with values around 25 $\mu\text{g}\cdot\text{g}^{-1}$ dry weight basis.²³⁻²⁷ The concentration of pVAC in the root and therefore available for absorption was much less for cassava than for sweet potato; however the daily intake by local populations might be greater for cassava than for sweet potato and this difference in daily intakes would consequently influence the respective impact of those biofortified crops.

Maize

In most of Africa, maize is harvested only once a year. Consequently, storage of maize grains is an important practice and a factor that could affect carotenoid retention. Maize grains are typically ground into maize meal to produce porridge, or cooked dough, and grits (samp). In West Africa, maize is commonly fermented after wet milling.²⁸ After milling, biofortified maize hybrids had high levels of carotenoid retention but these levels dramatically decreased when the product was stored.²⁹: the retention values were 105 and 137% in maize meal and samp respectively. The increase could be due to the higher extractability of maize flour compared to maize kernels. Burt *et al.*³⁰ reported that carotenoid retention in the whole grains of two maize genotypes was about 60% after 18 months storage at 25°C. The retention in grains of biofortified maize hybrids was 40% after six months of storage.³¹ Differences between the two studies could be due to differences in carotenoid composition of the initial maize.

In summary, pVAC were best retained in commodities (sweet potato, cassava, maize) when the crops were boiled/steamed in water. As the processing methods used became harsher on the food matrix (drying; frying etc.) pVAC retention was significantly lowered. Furthermore high carotenoid degradation occurred during storage of dried products (*e.g.* sweet potato, maize, cassava) at ambient temperature, and this constraint should be considered.

Iron and zinc

Iron exists under two different forms in food: haemic form in animal products and non-haemic form in plantfoods. Non-haemic iron, which is the main source of iron in developing countries, is generally poorly absorbed. Zinc and iron in plants are absorbed through the soil using similar pathways.³² It is important to consider not only the total content of iron and zinc in the crop but also the tissue localisation and specification (chelates; protein particles; etc.).³² Iron is stored in plants and animals as a protein called ferritin. A lot of the breeding effort has focused on targeting ferritin in order to increase iron content in biofortified crops.³³ Petry *et al.*³⁴ reported that understanding the influence of processing on release of the iron from the ferritin protein matrix is essential because it will influence iron bioavailability. Gibson *et al.*³⁵ reported that processes such as germination, fermentation or soaking are strategies to increase the bioavailability of minerals such as iron and zinc, since these processes will decrease the antinutrient levels that are present in cereals and pulses. It was reported that germination, fermentation and soaking resulted in improved bioavailability by reducing inhibitors such as phytates and polyphenols in plants in general.³⁶ Heat processing, by softening of the food matrix, can also release protein-bound minerals such as iron and hence facilitate their

absorption.³⁶ Little work has been reported on the effect of processing on the levels of iron and zinc in biofortified crops compared to carotenoids. Carvalho *et al.*³⁷ working on the cooking of common beans, showed that the levels of iron and zinc were partially lost in the broth. Pereira *et al.*³⁸ working on iron and zinc in cowpeas, showed that retention was high, around 90% (Table 1). Overall the iron and zinc retention were high for the cultivars of common beans (*Phaseolus vulgaris*) and cowpea beans (*Vigna unguiculata*), independent of the cooking methods applied (with/without soaking in water).^{37,38}

As opposed to provitamin A carotenoids, that are very susceptible to degradation by oxygen and temperature, minerals tend to resist degradation better because of their chemical nature. Processing may result in diffusion of the minerals in water and physical loss (i.e. in the cooking water) but in general processing is useful to increase mineral bioavailability because of the destruction of antinutrients that chelate the iron and zinc and reduce absorption.

On one hand, processing is beneficial to soften the food matrix which enhances provitamin A absorption and to destroy antinutrients which improves mineral absorption. On the other hand processing can also have a negative effect if the micronutrient content is significantly destroyed by extensive processing (i.e. pVACs) or by major physical loss in the cooking water for instance.

Dietary factors related to food matrix

The dietary factors have been reviewed focusing in particular on human studies that were conducted on conventionally and transgenic bred biofortified staple crops. Tables 2 and 3 describe the results of principal recent human studies conducted with biofortified crops on pVACs and minerals (iron and zinc) respectively. The dietary factors discussed below also include *in vitro* studies that were added to help understand the role of the food matrix.

Provitamin A carotenoids

Sweet potato

One of the first biofortified crops that was promoted against VAD as part of international programmes has been sweet potato. Promotion of orange-fleshed sweet potato was initiated in 2006 as part of the 'HarvestPlus Challenge Program Reaching End Users'. Prior to this, a bioconversion study by Haskell *et al.*³⁹ reported a bioconversion factor of ~13:1 from biofortified sweet potato puree, however the methods, based on changes in vitamin A stores to estimate bioconversion factors, are different from the current methods that collect triacylglycerol-rich lipoprotein plasma (TRL) straight after food consumption and the results may not be comparable and as accurate. Medium to large human intervention studies (Table 2) with biofortified sweet potato started in 2005 with the assessment of VA status of children using a Modified Relative Dose Response (MRDR) test to determine the adequacy of liver vitamin A stores.⁴⁰ Van Jaarsveld *et al.*⁴⁰ showed greater improvement in VA liver stores in the treatment group (OFSP) than in the control group (White fleshed sweet potato (WFSP)): 87% against 78%, respectively. However the low percentage of children with inadequate liver store at baseline was a limitation of the study.

Other large-scale trials were conducted as 2 year intervention programs in Mozambique⁴¹ and Uganda⁴² to introduce OFSP to local populations. These two interventions were targeted on

improved VA intakes from OFSP and therefore are not listed in Table 2. Prevalence of low serum retinol was around 10 % lower in children in these two studies, but there was no overall improvement on serum retinol or VAD prevalence among women. One randomized controlled trial to assess the effect of daily consumption of OFSP in 120 Bangladeshi women over 60 days was reported.⁴³ Despite an increase in plasma β -carotene, the impact of OFSP on VA status was not significant and suggested that bioconversion of BC to VA was limited in this population. These three studies focused on VA status improvement and showed a low to modest impact on VA status but none of them tested for bioconversion factors.

Altogether these results would have been needed to be strengthened by post prandial trials to determine the bioavailability and bioefficacy of BC from OFSP. There was however a consensus that BC bioaccessibility from boiled OFSP is very low (0.5-9.0%).^{43-44,45} In spite of the low BC bioaccessibility, the addition of fat was shown to significantly enhance bioaccessibility: Bechoff *et al.*⁴⁶ showed that, the percentage of micellarised all-trans-BC after *in vitro* digestion was greater in products cooked with oil - chapati (73%) and mandazi (49%) - as compared with the boiled ones - porridge (16%) and purée from boiled root (10%) . In addition, the high content of BC in OFSP can offset the relatively poor bioaccessibility, as this was demonstrated by the efficacy of bioconversion in Mongolian gerbils (6.5:1).⁴⁷ More *in vivo* studies to assess the influence of the amount and type of fat on bioavailability in OFSP preparations would be needed.

Cassava

Human interventions with biofortified cassava were very different in their approach to those with sweet potato. Three recent and easily comparable studies were conducted in Colombia and in the USA with healthy women to measure changes in the triacylglycerol-rich lipoprotein fraction (TRL) in plasma.⁴⁸⁻⁵⁰ Globally these post prandial studies showed very efficient bioconversion factors from 2.3 to 4.5 after ingestion of porridge or gari made with biofortified cassava.

The amount of dietary fat did not appear to affect the bioconversion factor because a high efficacy was obtained after consumption of a low fat porridge.⁵⁴⁸ Similar results were found in a second post-prandial trial in USA, where no significant differences were observed in bioconversion factors (4.2:1 and 4.5:1) when volunteers consumed porridges, containing 20 g or 6g of fat, respectively.⁴⁹ These results seemed to demonstrate that a small amount of fat, around 3-10g, was sufficient to substantially increase carotenoid bioavailability.^{51,52} In a more recent study by Zhu *et al.*⁵⁰, the quality of dietary fat seemed have an impact on the bioconversion factor: ingestion of biofortified cassava (BFC) gari and white gari with red palm oil (RPO) increased the carotenoid and retinyl palmitate concentrations in TRL plasma. It should be noted that the authors compared RPO gari with a gari from BFC containing the same amount of fat (10g) but the food matrix differed (BFC or RPO on white cassava). BC from red palm oil was more bioavailable than the BC from biofortified cassava and this might be because the former was completely emulsified in the fat. However, bioconversion factors for gari made with RPO and from BFC (2.3 ± 0.3 and 4.2 ± 1.5 , respectively) were not significantly different and were highly variable in all groups, presumably because of genetic differences.

Overall, the results from these studies showed that preparation of porridge or gari from BFC increased carotenoids and retinol palmitate in TRL plasma concentrations in well-nourished

women and support evidence that biofortified cassava is effective in food-based interventions. The bioconversion factors of approximately 3:1 or 4:1 observed in these studies were relatively efficacious compared with data from fruits and vegetables in the range of 3.6-28:1.⁶ Moreover, these data on human studies seem to validate *in vitro* studies reporting the relatively high bioaccessibility of BC from boiled cassava (25-30%) or from gari (30-37%) despite the low BC retention during gari processing for the latter.^{53,54} Other *in vitro* studies confirmed that addition of fat by a frying process increased BC bioaccessibility.^{55,56}

A recent study measuring the impact of boiled and pureed cassava consumption by Kenyan children⁵⁷ showed a more modest impact on vitamin A status based on the modest increase in serum retinol. However there were critics from this study expressing concerns about the validity of the results when serum retinol concentration is the primary biological indicator of vitamin A status in intervention studies⁵⁸ and other biomarkers such as nightblindness, dose-response tests to evaluate total body stores (TBR) etc. were advised to be included.

Maize

Three human interventions were carried out in 2010, 2011 and 2014 with BC-biofortified maize used in porridge preparation.⁵⁹⁻⁶¹ Data from these studies concluded that biofortified orange maize (BM) is an effective source of vitamin A. Methodologies (TRL plasma, serum concentration, TBRs), design (from 6 to 130 volunteers), gender, and age of the subjects (children, men, women) were very different in the three studies.

The study by Li *et al.*⁵⁹ was a post-prandial study conducted on 6 healthy women consuming BM porridge. The authors found a VA equivalence of 6.5:1 on average. A higher efficiency of absorption of the vitamin A reference dose ingested with white maize porridge was observed, with a bioconversion factor of 2.34:1. The difference in bioconversion can be explained by the difference in food matrix: in the reference meal, the BC was dissolved in oil (reference), and in the test meal it was part of the vegetable matrix (in BM). Nonetheless the VA equivalence value (6.5:1) was a good factor relative to the average standard value of 12:1.⁶² Moreover the bioconversion factor of 6.5 was a mean of values from 3.8 to 13.32 (coefficient of variation of 54 %). One limitation of the study was the low number of subjects, which led to a large inter-individual variability. Overall the authors showed that 250g porridge would provide 15 % of the estimated requirement (500 µg RAE/day) for adult women.⁵⁹

The second human study measured the serum retinol concentration of 8 healthy men in Zimbabwe over a 36 day period.⁶⁰ After consumption of BM-porridge containing 1.2 mg of labelled BC, the average conversion factor was 3.2:1 and this was different from the first study (6.5:1). The difference in amount of dietary fat in the porridges (20.5g versus 8g in the first study) may explain the better bioconversion efficiency. Although the method and design used differed, both human studies showed that BC from BM in porridge was converted efficiently to VA.

A recent large-scale trial designed to determine changes in total body reserves (TBRs) of vitamin A with consumption of BM porridge was conducted.⁶¹ Zambian children aged 5-6 year-old were randomly assigned to eat either a white or BM maize porridge meal at breakfast and lunch for 6 days per week over 90 days. TBRs were higher in the orange maize group compared to the control group and bioconversion factor was estimated to be 10.4:1. This bioconversion factor was not as good as the precedent studies but it was calculated indirectly from the change

in TBRs and also obtained from a mostly non-healthy child population (52% were stunted and or undernourished). Unexpected data on baseline estimates showed no VAD and high liver stores in the Zambian children and this raised uncertainties about the methodology to measure the children's status⁶³. The authors however explained that this population of Zambian children had been subjected to supplementation with preformed VA and this could have lead to hypervitaminosis and high liver store. These hypotheses were further confirmed by Tanumihardjo et al.⁶⁴ who showed that preformed VA from additional supplementation in VA from other interventions, and coinciding with the mango season, would have caused excessive total body stores and hence caused hypervitaminosis. No change in serum concentration was observed and this was probably because of homeostatic regulation. The measurement of biomarkers (high circulating concentrations of carotenoids and mildly elevated serum retinyl esters) confirmed high liver VA stores and therefore hypervitaminosis A in Zambian children⁶⁵. Specificity of serum concentrations to evaluate VAD was high but sensitivity of the serum retinol method requires improvement⁶⁶. *In vivo* studies on biofortified maize globally showed that BM was as efficient as a VA supplement to increase total body reserve in retinol and led to original and very interesting findings with regards to risk of hypervitaminosis when multiple international interventions were conducted^{61,64,65}.

Rice

Transgenic biofortified crops such as “golden rice” (GR) - developed in the last decade - are an approach, other than conventional breeding, to increase the level in pVACs or micronutrients (iron, zinc). Several years after its development by Ingo Potrykus and Peter Beyer, GR has overcome many hurdles and has now been incorporated in breeding programs although no country has yet approved it for use by farmers.⁶⁷ GR seemed to be an effective source of vitamin A in humans with an estimated conversion rate of BC to retinol of 2.3-3.8.^{68,69} Two studies were conducted by a USA research team with healthy American adults⁶⁸, and with Chinese children⁶⁹ using similar methodologies. Chinese children converted GR more efficiently (2.3:1) than did US adults (3.8:1). Differences were attributed to the age but mainly to the lower vitamin A status of Chinese children. These authors found less efficient bioconversion factors when children ingested spinach (7.5:1) compared to golden rice (2.3:1) and this was similar results found when BC was given in an oil capsule (2:1). It should be noted that the study⁶⁹ was retracted because of ethical concerns about the children's treatment during the trial.

A third more recent study evaluated the impact of triple-fortified rice (with BC, iron and zinc) on the VA status of school-children in Southern Thailand.⁷⁰ Results indicated that the mean TBRs in the children doubled after the two month intervention. Triple fortified rice was highly efficacious at improving VA status and demonstrated the usefulness of the paired stable isotope dilution technique used to assess VA status and measured TBRs.

The efficient bioconversion of BC from GR observed in these human trials may be due to its simple cell structure in the food matrix that would possibly make it more digestible⁶: contrary to green leafy vegetables where carotenoid pigments are embedded in chloroplast structures, which contain insoluble fibres that decrease bioavailability, carotenoids in the rice food matrix are embedded in chromoplasts with less of such fibres. In addition, chromoplasts in the golden rice are not in a crystalline form such as in carrot but in a globular form that is more bioaccessible. Finally, rice contains amylose and amylopectin with a high starch digestibility, which also enhances carotenoid bioavailability.

Banana

Another crop produced using transgenic technology is “super banana”. Due to the sterile triploid nature of the banana, transgenic breeding was conceived and the research was conducted by Queensland University of Technology supported by the Bill and Melinda Gates Foundation. A collaboration with the National Agricultural Research Organisation in Uganda was built to develop biofortified banana to tackle two major micronutrient deficiencies: iron and vitamin A.⁷¹ The first human trial examining the efficacy of the super banana was approved in the US in 2014.⁷² A human bioavailability study is currently underway and pro-vitamin A bananas are expected to be released in 2019.

Globally, human studies given in Table 2 showed that bioavailability of pVACs in biofortified crops was mainly affected by the food matrix and the type of crop. Very efficient bioconversion factors from 2.3:1 to 4.5: 1 for golden rice and cassava suggest that these crops had more easily digestible food matrices. Further human studies are needed on biofortified maize to calculate the bioconversion factor more accurately (currently there is a wide variability: 3.2:1 to 10.4:1 in the different studies). The modest efficacy impact of OFSP suggests that carotenoids were less extractable from that matrix than from other biofortified crops. Overall conducting more efficacy studies is advisable in order to determine bioconversion factors from biofortified crops with various levels of fat addition.

Iron and Zinc

Eight recent human studies were identified on four biofortified crops containing higher iron or zinc concentration (Table 3): three studies were reported on pearl millet, two on beans, two on rice and one on wheat. Of these studies, only two were conducted with zinc biofortified crops.

Iron and zinc strongly interact with their chemical environment in the plant and also with other components during the passage through the human digestive system. Iron and zinc bioavailability in staple foods were generally low because of the highly complex chemical interactions with other compounds⁷³: enhancers or inhibitors had a great influence on mineral bioavailability. The major inhibitor was phytate, which chelates iron or zinc and formed insoluble complexes. Other more minor antinutrients could be polyphenols such as tannins, and fibres. Conversely ascorbic acid was a good enhancer of iron because it reduced iron from the diet to ferrous iron, a form absorbable by the intestinal cells. Other compounds such as sulphur amino-acid or organic acids could also promote zinc absorption.⁷⁴

Pearl millet (iron and zinc)

A recent study showed a significant improvement of iron status in Indian iron-deficient children after 6 months of feeding with biofortified pearl millet (BPM) as unleavened bread (bhakri).⁷⁵ Two other studies conducted using stable isotopes on pearl-millet showed an improvement in total iron and zinc absorption.^{76,77} Consumption of iron-biofortified millet meals by young Beninese women doubled the amount of iron absorbed comparatively with a regular millet meal⁷⁶. Likewise, consumption of iron and zinc biofortified pearl millet by 2 year-old Indian children resulted in a significant increase in iron and zinc absorbed⁷⁷. Bioavailable amounts of these minerals were enough to meet nutritional requirements of those children⁷⁷.

Beans (iron)

Two similar studies on beans provided clear evidence that phytic acid was the major inhibitor of iron absorption.^{78,79} In the first study⁷⁸ the iron absorbed from the control and from high iron beans (HIB) was not different despite both meals having equal molar phytic acid (PA): iron ratios. In the second study⁷⁹ there was an improvement in iron absorption when an HIB meal was consumed (19% higher than the control), and with dephytinisation, the quantity of iron absorbed increased by 37% and 51% for ~50% and >95% dephytinisation, respectively.

Rice and wheat (zinc)

Similar improvements in bioavailability are reported on zinc biofortified crops such as rice and wheat.⁸⁰⁻⁸² With regard to zinc status, Haas *et al.*⁸⁰ reported a 20% greater increase in the ferritin and body iron levels of nonanemic filipino women after consuming biofortified rice daily. According to Islam *et al.*⁸¹ total zinc absorption did not differ among Bangladeshi children when they ate conventional rice or higher-zinc rice. However the lack of difference may be due to phytate levels that inhibit zinc absorption. The authors highlighted the need of a rice cultivar with higher zinc and lower phytate. Further results on high zinc wheat indicated that potential increases in zinc absorption from biofortified wheat could be achieved by reducing the level of phytate by removal of the bran and germ.⁸²

Polyphenols such as tannins are another inhibitor of absorption. Petry *et al.*⁸³ initially suggested that polyphenols were the main factor contributing to the low iron bioavailability from common bean however in a later study, the authors³¹ showed that bean polyphenols played in fact a minor role compared with phytic acid *in vivo*. Polyphenols had a clear inhibitory effect on animal model studies: a study of Tako *et al.*⁸⁴ showed that there was not a clear improvement in iron status in poultry fed with high iron beans. In this study, there was clear evidence that polyphenolic compounds present in the seed coat in black beans had a strong inhibitory effect on iron bioavailability⁸⁴. In an *in vitro* study on beans, Ariza-Nieto *et al.*⁸⁵ showed that polyphenols had had greater inhibitory effects on iron bioaccessibility as compared to phytates, however the opposite was found *in vivo*³⁴.

Bioavailability of minerals is significantly reduced mainly by phytates but also by other antinutrients such as tannins. More *in vivo* studies are needed to understand the role of ascorbic acid as an enhancer of bioavailability in biofortified crops.

Some of the *in vivo* studies showed that a combination of mineral and pVAC biofortifications in a crop seemed more efficacious than a single micronutrient and this underlines the importance of understanding the interaction of micronutrients and their relation to human status.

Human factors

Status/interaction

VAD and ID or ZD often occur together as a consequence of dietary pattern and social factors but also as a result of possible interactions between micronutrients or micronutrient regulation, and are influenced by the general status of the host in relation to infection and disease.

Many intervention studies have focused on understanding the links between vitamin A intake and iron status: it was shown that vitamin A supplementation had an effect on blood hemoglobin concentrations.⁸⁶ VAD was first associated with iron metabolism and reduced incorporation of iron into erythrocytes in rats.⁸⁷ In a human study, VAD was related to erythropoietin synthesis, and transcription of many hepatic genes.⁸⁸ In addition, it was shown that VA status could have an impact on the synthesis or catabolism of proteins involved in hepatic iron storage and mobilisation.^{89,90} A recent study by Htet *et al.*⁹¹ demonstrated the interdependence of vitamin A and iron status in contributing to anaemia. The authors reported that better iron status was associated with a higher serum retinol concentration only in the ID group. They concluded that improvement in VAD was required to reduce ID. According to Michelazzo *et al.*⁹² it seemed that simultaneous use of vitamin A and iron supplements was more effective to prevent iron deficiency than the use of one micronutrient alone. On the other hand little is known about the effects of iron on vitamin A metabolism and status. It seems that ID affected vitamin A by impairing the mobilization of VA stores in the liver, leading to lower serum retinol concentration and an increase in hepatic retinoids. These changes were attributed to a reduction of retinyl ester hydrolases.⁹³

The relationship between vitamin A and iron depends also on many factors such as the presence of inflammation, acute or chronic diseases. The prevalence of infection and multiple nutrient deficiencies may affect the micronutrient bioavailability in developing countries. It should be noted that an increase in serum retinol concentration may be dependent on the degree of helminthic infection.⁹⁴ Parasite infections may decrease the impact of zinc or vitamin A supplementation on children and impair childhood growth.⁹⁵ In addition there are some limitations in using serum retinol concentration as an indicator of vitamin A status because it is decreased by acute and underlying chronic infections^{58,94}.

Oliveira *et al.*⁹³ reported a possible interaction between zinc and iron in studies where supplementation with zinc and iron combined was less effective than supplementation with zinc or iron alone. This is because zinc and iron have similar absorption pathways, which may cause competition between these two minerals during their absorption.

Genetic factors

Genetic variation is probably a key factor explaining the large inter-individual variability observed in the bioavailability and bioconversion of pVACs into VA. (Borel *et al.*⁹⁶). In the last decade, this has led some researchers to divide subjects into a low and high responder phenotype.⁹⁷ The enzyme responsible for BC conversion into retinal is β -carotene 15,15'-monooxygenase (BCMO1). Explanations on genetic polymorphisms contributing to the poor converter phenotype were first reported by Leung *et al.*⁹⁸. A range of Single Nucleotide Polymorphisms (SNPs) within the BCMO1 coding region were identified and revealed that catalytic activity of the enzyme could be reduced by approximately 48 -59 % .⁹⁹ Furthermore, these SNPs were responsible for large inter-ethnic variations with SNP frequencies varying

from 19 to 100 %. A recent discovery indicated that proteins involved in carotenoid metabolism, mainly in uptake and transport by intestinal cells (SR-BI (scavenger receptor class B type 1), CD36 (cluster determinant 36), NPC1L1 (Niemann Pick C1-like 1) or the ATP-binding cassette transporters (ABC) transporter (transmembrane proteins)) could be associated to genetic variants in genes encoding for these proteins.⁹⁶ Finally a recent study of Borel *et al.*¹⁰⁰ showed that 25 SNPs in 12 genes explained 69 % of the variance in the post-prandial chylomicron β -carotene response. Amongst the genes, the ABC transporter family and BCMO1 (actually named BCO1) were effectively indirectly or directly associated with the post-prandial chylomicron β -carotene response.¹⁰⁰ These findings demonstrate that genetic variability should be included into future studies because it can influence the effectiveness of biofortified crops to improve micronutrient status in different ethnic groups from developing countries.

Conclusion

We have explored the influence of dietary factors and human factors on micronutrient bioavailability in biofortified crops. Although dietary factors have now been well identified and studied, human factors require more investigation given the complexity of interactions.

With regards to VAD research, there is a need for more studies to be carried out with local populations in developing countries to investigate whether nutritional status, genetic factors, and diseases might influence vitamin A equivalency of BC in biofortified crops. Future isotopic labelling studies should be carried out to obtain more accurate data for various factors influencing the bioconversion. The importance of genetic factors on bioavailability is a recent discovery that was made possible by showing polymorphism in the BCMO1 gene and this recent advance looking at other genes involved in carotenoid bioavailability seems promising. Human studies to understand BCMO1 gene polymorphism across different ethnic groups should be carried out.

Due to expensive isotope methodology, and complex chemical interactions, mineral bioavailability studies are yet in their infancy and are still extremely challenging. This complexity of the methodologies explains in part why there are very few human studies looking at iron and zinc bioavailability from biofortified crops. Human intervention on iron biofortified pearl-millet and bean showed encouraging results while those on biofortified rice and wheat require efficacy evidence, in particular on zinc bioavailability and status. Indicators of individual micronutrient status must be refined in order to understand the micronutrient synergies/antagonists.

Globally, more efficacy trials and effectiveness studies are needed on micronutrient absorption in biofortified crops taking into account human status and inter-individual variation linked to genetic factors.

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Table 1. Retention of provitamin A carotenoids and minerals from biofortified crops during processing and storage

Table 2. Conversion efficiency of β -carotene from biofortified crops to retinol and efficacy of provitamin A biofortified crops (sweet potato, cassava, maize, rice)

Table 3. Percentage absorption and amount absorbed of zinc and iron, and molar ratio of phytic acid (PA) on zinc or iron from biofortified crops (pearl millet, beans, rice, wheat) compared to control