1 **Biofortification of crops with nutrients: factors affecting utilization and storage**

2 Joana Díaz-Gómez [1], Richard M Twyman [2], Changfu Zhu [3], Gemma Farré [3], José C E

3 Serrano [4], Manuel Portero-Otin [4], Pilar Muñoz [3], Gerhard Sandmann [5], Teresa Capell [3]

4 and Paul Christou [3, 6]

5

6 **Abstract**

7 Biofortification is an effective and economical method to improve the micronutrient

8 content of crops, particularly staples that sustain human populations in developing

9 countries. Whereas conventional fortification requires artificial additives,

10 biofortification involves the synthesis or accumulation of nutrients by plants at source.

11 Little is known about the relative merits of biofortification and artificial fortification in

12 terms of nutrient bioaccessibility and bioavailability, and much depends on the

13 biochemical nature of the nutrient, which can promote or delay uptake, and determine

14 how efficiently different nutrients are transported through the blood, stored, and

15 utilized. Data from the first plants biofortified with minerals and vitamins provide

16 evidence that the way in which nutrients are presented can affect how they are

17 processed and utilized in the human body. The latest studies on the effects of the food

18 matrix, processing and storage on nutrient transfer from biofortified crops are reviewed,

19 as well as current knowledge about nutrient absorption and utilization.

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21 **Addresses**

22 [1] Department of Food Technology, University of Lleida – Agrotecnio Center, Lleida,

23 Spain

24 [2] TRM Ltd, York, UK

25   [3] Department of Plant and Forestry Science, University of Lleida – Agrotecnio Center,

26   Lleida, Spain

27   [4] Department of Experimental Medicine, University of Lleida–Biomedical Research

28   Institute of Lleida (IRB Lleida), Lleida, Spain

29   [5] Biosynthesis group, Department of Molecular Biosciences, J. W. Goethe University,

30   Frankfurt, Germany

31   [6] ICREA, Catalan Institute for Research and Advanced Studies, Barcelona, Spain

32

33   Corresponding author: Christou, Paul (christou@pvcf.udl.cat)

Introduction

Nutrients in the human diet ultimately come from plants, but all our major food crops lack certain essential micronutrients (vitamins and minerals) [1]. The endosperm of cereal staples such as rice, wheat and maize are the most important source of calories for humans, providing ~23%, ~17% and ~10% of total global calories, respectively [2]. However, endosperm tissue lacks sufficient amounts of vitamins (particularly vitamins A, E, C and folate) and minerals (particularly iron, zinc and selenium) [1,3]. Iron and zinc deficiencies affect more than 50% of the human population, resulting in poor growth and development, an impaired immune system, fatigue, muscle wasting, sterility and even death [2,3]. More than 4 million children worldwide suffer from severe vitamin A deficiency (VAD), including 250,000–500,000 per year who become partially or totally blind [4]. Women have a higher demand for vitamin A during pregnancy, and currently more than 20 million pregnant women in developing countries suffer from VAD [4].

Strategies to address micronutrient deficiency include dietary diversification, nutritional supplements, fortification and biofortification [1–3]. A combination of approaches is likely to provide the greatest overall benefit, but in some populations dietary diversification is impractical and supplements are only suitable as short-term interventions [2,3]. Fortification requires the addition of nutrients to food products, e.g. iodine is added to table salt, and iron, zinc and folate are added to flour to make bread [2,3]. One major drawback of these approaches is the limited stability of the additives, e.g. folate added to rice becomes more soluble at higher temperatures and is lost when the rice is boiled [2]. A second disadvantage is that additives can also affect the quality of food, e.g. iron additives are oxidized over time and this has an impact on taste [3]. The third and major limitation of conventional fortification is that it is mainly suited to

developed countries with the necessary technical infrastructure and distribution networks, but is less appropriate for developing countries with their extensive reliance on subsistence agriculture [2]. Biofortification can address all three issues by facilitating the development of nutrient-dense staple crops that can be grown and distributed using existing agricultural practices [3,5].

Biofortification is well established in principle but there are few practical examples of deployment thus far. Zinc-enriched rice and wheat have recently been deployed in Bangladesh and China, respectively; an orange sweet potato rich in provitamin A carotenoids has been released in Mozambique and Uganda; and provitamin A rich maize has been released in Zambia and Nigeria [5]. Golden Rice II, the first transgenic biofortified crop engineered with provitamin A carotenoids in the endosperm, has incurred multiple delays in terms of deployment. It is currently being backcrossed into locally adapted varieties in the Philippines, Indonesia, India and Bangladesh [5]. Multivitamin corn (registered as the protected variety Carolight® in Spain) was developed by transforming an elite white-endosperm South African inbred line with four genes representing three different vitamin biosynthesis pathways, increasing the levels of β-carotene, other carotenoids, vitamin C and folate [6]. Carolight® also contains a *Bacillus thuringiensis* (*Bt*) gene making it pest resistant [7]. Biofortification is a sustainable approach which can bring nutritious staple crops to populations that are difficult to supply with supplements or fortified food products, and once the crop is developed there are no recurring costs other than those associated with normal agriculture. However, it is necessary to consider the efficiency of nutrient delivery by biofortified crops compared to other interventions in order to determine the long-term benefits of this approach. Data from the first biofortified crops are now available to allow such comparisons.

84

Fate of nutrients produced in plants

The fate of organic nutrients in plant tissues is highly dependent on their solubility and their affinity for the constituents of the plant tissue matrix.

Folate

Folate is soluble in water and is easily released from the matrix, thus plasma folate levels are higher following the consumption of minced/chopped spinach rather than whole leaves both as raw tissue [8] and after microwaving [9]. Dietary fibers such as cellulose, lignin, pectin and alginate do not appear to affect folate bioavailability [10]. Baking causes the loss of endogenous bread folates (~40%) as well as added synthetic folic acid (30–60%). Furthermore, the bread matrix inhibits folate absorption [11].

Carotenoids

In contrast, the bioavailability of fat-soluble nutrients appears to be much more dependent on associations with matrix components and other dietary constituents, as shown for the six major dietary carotenoids (β-carotene, α-carotene and β-cryptoxanthin with provitamin A activity, lycopene, lutein and zeaxanthin without provitamin A activity) [12]. Carotenoids are associated with proteins in many green leafy vegetables, whereas in carrots and tomatoes they are also stored in a semi-crystalline form [13,14]. Cooking, food processing, and the enzymatic processes during digestion weaken the cell walls and disrupt the protein-carotenoid complexes, promoting release and increasing bioavailability [15]. The bioavailability of carotenoids appears to depend on food particle size, with more efficient absorption from smaller food particles produced by homogenization, grinding, or milling. The bioavailability of carotenoids after release is favored by the presence of fats because carotenoids are incorporated into lipid droplets

108 before entering the micelles, whereas soluble fibers, sterols and stanols inhibit the

109 absorption of carotenoids [13,16–18]. The inhibitory effect of fibers may reflect the

110 higher viscosity of fibrous solutions, the formation of gel aggregates, the incomplete

111 hydrolysis of triacyl glycerols, or carotenoid aggregation [19]. Carotenoids are

112 lipophilic and may also compete with plant sterols and stanols for solubilization in

113 mixed micelles [18].

114 *Vitamin E*

115 Vitamin E comprises eight fat-soluble molecules (α, β, γ and δ tocopherol and the

116 corresponding tocotrienols, with α-tocopherol possessing the greatest biological

117 activity) and like carotenoids its bioavailability is therefore highly dependent on

118 interactions with the food matrix [20,21]. Accordingly, the bioaccessibility of vitamin E

119 varies extensively in different types of food, ranging from 0.47% in apple to almost

120 100% in banana, white bread and lettuce. Interestingly, the bioaccessibility of

121 α-tocopherol was similar to that of γ-tocopherol when sourced from almonds, wheat

122 germ, cheese or hazelnuts, but α-tocopherol was more bioaccessible than γ-tocopherol

123 when sourced from banana, bread, lettuce and milk. This may reflect the food matrix

124 effect, which determines the location of tocopherols, their physicochemical state, and

125 the co-presentation of absorption effectors such as fibers, fats, sterols and stanols [22].

126 *Calcium and iron*

127 The bioavailability of minerals is affected by the food matrix, intrinsic chemical

128 properties such as the oxidation state and counter-ion, and also by co-presented food

129 substances, because all of these factors can affect solubility [23]. Calcium must be

130 solubilized before it can be absorbed. The extracellular calcium concentration depends

131 on intestinal absorption, kidney reabsorption and bone resorption/formation, which are

132 regulated by the calcium sensing receptor (CaSR) located in the parathyroid gland [24].

133   The absorption of calcium is highly dependent on the abundance of phytate and oxalate,

134   which can combine with calcium to form insoluble complexes [25,26]. Calcium also

135   forms complexes with proteins, so cooking can help to release calcium for absorption,

136   but the cooking method is important because the soluble calcium leaches into water

137   used for boiling, but is retained during baking [27]. Vitamin D is also required for

138   calcium absorption [28]. Similarly, iron in meat and fish is relatively easy to absorb

139   because of its favorable oxidation state and its storage in the form of ferritin-iron

140   complexes that release the mineral readily, whereas some dietary proteins (such as

141   albumin, casein, phosvitin and conglycinin) and certain plant polyphenols can reduce

142   the bioavailability of iron [23,29].

143

144   **The role of the food matrix, food processing and storage**

145   *Food matrix*

146   The major role of the food matrix in terms of nutrient bioaccessibility and

147   bioavailability is to trap the nutrients within cells or subcellular compartments, and to

148   provide constituents that interact chemically with specific nutrients to either encourage

149   or delay their release, leading to their classification as absorption promoters and

150   inhibitors (Table 1). Lipid food components increase the bioaccessibility of fat-soluble

151   nutrients, so cooking methods that preserve fats (e.g. frying) tend to outperform

152   methods that disperse them (e.g. boiling) in terms of promoting the bioaccessibility of

153   nutrients such as β-carotene, as recently shown for biofortified cassava [30]. Similarly,

154   β-carotene bioaccessibility increased by 3–5 fold in a transgenic biofortified sorghum

155   line when the lipid content was increased from 5% to 10% [31]. Inhibitors such as

156   phytate, oxalate and polyphenols reduce the bioaccessibility of iron and zinc by forming

157   insoluble complexes. Transgenic maize, rice and sorghum with lower phytate levels in

158 the seeds have been developed to address this issue [32]. Biofortification is

159 advantageous for iron nutrition because plants can be engineered to maximize

160 bioaccessibility. In contrast, standard fortification is achieved using sparingly soluble

161 iron compounds to avoid an undesirable metallic taste, but the bioavailability of such

162 compounds is low [33]. Agronomic interventions are short-term strategies that focus on

163 the use of soil and foliar mineral fertilizers, but regular applications are required [34]. In

164 maize, rice and wheat, foliar fertilization achieves higher levels of zinc accumulation

165 than soil fertilization [35]. Mineral biofortification is most efficient when cereals are not

166 consumed as flours, e.g. rice grain. Accordingly, zinc in rice grains biofortified using

167 zinc-rich fertilizer is absorbed to a similar extent as the same rice variety fortified

168 artificially with zinc immediately before consumption [36].

169 *Food processing*

170 Food processing can enhance the bioaccessibility and bioavailability of nutrients by

171 removing inhibitors or releasing nutrients from the food matrix (Table 2) but it can also

172 reduce nutritional value. For example, most cereal grains are dehulled and milled before

173 consumption, causing significant losses of minerals [32] and certain vitamins [37].

174 Carotenoid levels tend not to be affected by light milling, but greater losses are caused

175 by heavy milling [38,39]. Genetic engineering strategies that promote the accumulation

176 of nutrients in the endosperm rather than the bran or husk are therefore useful to

177 increase the nutritional value of polished grains [40], as shown by the expression of

178 enzymes that promote the synthesis of phytosiderophores in rice, leading to the

179 modulation of endogenous metal transporter gene expression and the mobilization of

180 zinc and iron from the bran to the endosperm [41]. Cooking and thermal food

181 processing methods such as pasteurization can destroy heat-sensitive organic nutrients

182 such as folate and B6 group vitamins, and the cooking method can also encourage

183 leaching, but the bioaccessibility of other nutrients can increase when they are released

184 from the plant matrix by cooking. Transgenic biofortified cassava provides sufficient

185 bioavailable vitamin B6 after cooking: the leaves and roots retain, respectively, 9-fold

186 and 4-fold more non-phosphorylated B6, than non-transgenic cassava [42]. Transgenic

187 biofortified rice meets folate requirements even after cooking losses of 45% (100 g of

188 rice contains 500 µg of folates) [43]. Interestingly, only 43% of the original content of

189 provitamin A carotenoids was retained in fortified rice grains after cooking, whereas

190 iron, zinc, folic acid and vitamin B12 levels usually remained above 80% of the original

191 value [44]. In contrast, β-carotene was retained when biofortified maize [38,39] and

192 biofortified pumpkin [45] were cooked (>72% and >78%, respectively) suggesting that

193 biofortification prevents the loss of provitamin A carotenoids during cooking more

194 effectively than standard fortification, probably due to the food matrix effect. In hens

195 fed on transgenic biofortified maize, provitamin A carotenoids are preferentially

196 diverted to the liver, whereas non-provitamin A carotenoids accumulate in the egg, in

197 some cases doubling the initial concentration in the feed. When non-provitamin A

198 carotenoids were supplied as intrinsic components of the transgenic biofortified maize,

199 these nutrients were more efficiently absorbed than carotenoid additives in the standard

200 commercial maize diet [46]. Transgenic biofortified cassava also preserves the

201 bioaccessibility of provitamin A carotenoids after processing, with a greater efficiency

202 of β-carotene transfer to micelles (30–45%) than non-transgenic cassava (27–31%) [47].

203 In contrast, the transfer of β-carotene to micelles in transgenic sorghum was less

204 efficient (1–5%) than in non-transgenic sorghum (6–11%) [31]. Several studies have

205 highlighted the importance of genotype-specific effects on the retention of carotenoids

206 during identical processing treatments, probably reflecting differences in the food

207 matrix [30,38,45,47]. The impact of cooking on the retention of β-carotene also varies

208 according to the genotype [30,38,45], and genotype has a greater effect on the quantity

209 of β-carotene in the micelle fractions than on the retention of β-carotene after processing

210 [47].

211 *Storage*

212 The stability of nutrients during storage is also an important consideration because

213 biofortified maize loses a greater quantity of carotenoids during post-harvest storage

214 than during cooking [38]. As discussed above for cooking and processing, genotype has

215 an important impact on carotenoid stability during storage [48]. Moreover, maize

216 genotypes which lose more carotenoids during drying tend to lose less during storage

217 [49]. Biofortified cassava was more susceptible to carotenoid losses during storage than

218 white cassava with added red palm oil, suggesting that fatty acids can prevent

219 carotenoid degradation [50]. Transgenic biofortified rice with improved folate stability

220 was recently reported to maintain folate levels for 4 months when stored at 28ºC [43].

221 The sequestration of carotenoids in chromoplasts, which act as a metabolic sink, can

222 enhance carotenoid levels during storage, as recently shown for transgenic potatoes (cv.

223 Désirée) in cold storage for 5 months [51]. Nevertheless, when the Phureja cultivar was

224 used as the parental genotype (high carotenoid content in tubers) instead of Désirée (low

225 carotenoid content in tubers), there were no significant changes in total carotenoid

226 levels during cold storage [52].

227

228 **Downstream behavior of absorbed nutrients**

229 Nutrient supplements and fortified foods are provided in well-controlled doses to avoid

230 toxicity. One concern about biofortification is that dosing would be more difficult to

231 control, but recent studies have shown that the uptake of nutrients from biofortified

232 crops is regulated at the level of absorption from the gut, and also at the cellular level

233 and by the modulation of storage reservoirs, based on the abundance of nutrients already

234 in the body and the demand for certain nutrient molecules [53].

235 Each vitamin and mineral has a specific transporter that facilitates its uptake from the

236 gut, but some unrelated nutrients can also share the same transporter, as shown for the

237 sodium-dependent multivitamin transporter that can mobilize pantothenic acid, biotin,

238 α-lipoic acid and iodine [54]. In this context, the transport of one nutrient can be

239 inhibited in a concentration-dependent manner by other compounds that share the same

240 transporter. Fat-soluble compounds are also mobilized by lipid transporters that vary in

241 specificity. For example, carotenoids are absorbed via scavenger receptors (class B type

242 1 and Niemann-Pick type C1-like 1) that are selective for particular carotenoid

243 molecules such as lutein [55]. Some nutrients can only be absorbed as a complex with a

244 ligand that is secreted into the gut. For example, intrinsic factor is secreted by gastric

245 parietal cells to absorb cobalamin (vitamin B12), polyglutamyl folates must be

246 processed by glutamate carboxypeptidase II, and the pancreatic secretion of γ-glutamyl

247 hydrolase is necessary to release folate for absorption [56].

248 The intestinal uptake of nutrients is adaptively regulated by the substrate level in the

249 diet and depends primarily on the number of transporters in the apical and basolateral

250 cell membranes of endothelial cells. For thiamin, this involves the transcriptional

251 regulation of thiamin transporter-2 [57]. The production of nutrient-specific transporters

252 is regulated at the level of transcription. High levels of nutrient bioavailability lead to

253 the suppression of transcription and starvation causes the transporter gene to be induced.

254 In some cases, specific epigenetic changes have been observed in the promoter of the

255 transporter gene, e.g. the oversupply of riboflavin leads to the epigenetic suppression of

256 the riboflavin transporter gene [58].

257   The transport of nutrients after absorption may also be regulated. For example,

258   fat-soluble vitamins, carotenoids and ω-3 fatty acids (particularly docosahexaenoic and

259   eicosapentaenoic acids) are transported in lipid vesicles that require chylomicron

260   assembly and secretion, and these processes are inhibited when the corresponding

261   nutrients are plentiful [59]. Similarly, iron is exported from enterocytes via ferroportin

262   and its distribution is limited by the availability of transferrin. Finally, specific

263   intracellular sensors of nutrient bioavailability may regulate tissue distribution. For

264   example, the absorption of iron is inhibited by the regulatory protein hepcidin which is

265   stored in macrophages. Interestingly, hepcidin synthesis is sensitive to both circulating

266   iron and intracellular iron stores because the macrophages communicate with

267   hepatocytes to regulate hepcidin release via multiple indicator proteins, including

268   transferrin and transferrin receptor-2 [60].

269

270   **Conclusions**

271   The biofortification of staple crops was envisaged as a sustainable strategy to deliver

272   nutritious food to populations that are unsuitable for other intervention measures, but

273   the bioavailability of nutrients in biofortified crops must be confirmed before they can

274   be widely deployed. The bioavailability of nutrients is partly dependent on the intrinsic

275   qualities of each nutrient molecule and partly dependent on their presentation in the

276   context of the food matrix.

277   The major difference between biofortification and standard fortification is that the latter

278   involves additives that are mixed with the food, whereas biofortification embeds the

279   nutrients inside plant cells. The bioencapsulation of nutrients in this manner can prevent

280   them from leaching during cooking and processing, as shown by the direct comparison

281   of β-carotene levels after cooking fortified and transgenic biofortified rice, but can also

282 enhance the binding of nutrients to plant proteins and fibers, as shown for iron and other

283 minerals. The full value of biofortified crops can therefore be realized only by

284 combining the adoption of biofortified varieties with the most appropriate food

285 preparation and cooking methods to maximize the bioavailability of different nutrients.

286 Moreover, cooking and storage losses could be reduced by growing crops in which the

287 nutrients are more stable (e.g. transgenic folate-biofortified rice). Biofortified crops can

288 help to alleviate micronutrient deficiency in at-risk populations in a sustainable manner.

289 Some biofortified crops (e.g. rice, maize, cassava and pumpkin) achieve better results

290 than others (e.g. sorghum), but rural populations are accustomed to eating staple crops

291 commonly harvested in their area, so biofortification strategies must be tailored for

292 different communities to achieve the greatest improvements in nutritional health.

293

299

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Table 1. Relationship between micronutrients and the food matrix.

Carotenoids [14,26]
• In photosynthetic plant tissues, carotenoids are bound to proteins in the inner chloroplast membrane, whereas in other tissues, such as fruits and endosperm, they are mainly found in chromoplasts. Carotenoids accumulate in the plastoglobuli of chloroplasts and chromoplasts, but in the latter they are also deposited as crystals. • Cell walls, carotenoid-protein complexes and fibers trap carotenoids and inhibit absorption. • The type and amount of fat can influence carotenoid absorption by promoting the excretion of bile salts, enhancing micelle formation and carotenoid solubilization. • Xanthophylls are more hydrophilic than carotenes and are thus easier to absorb. • Carotenoid *cis* isomers are more easily absorbed than *trans* isomers due to their greater polarity and solubility.
Iron and zinc [32,33,49,65]
• Iron and zinc are chelated by other food components for transport (e.g. nicotianamine, peptides, proteins and organic acids). Proteins can also enhance absorption. • Heme iron and organic zinc complexes are more readily absorbed than non-heme iron and inorganic zinc salts. The absorption of non-heme iron and zinc from plant-based foods can be enhanced by consuming meat, poultry, fish or seafood in the same meal. • Phytate, oxalate, phenolic compounds and fibers form insoluble complexes with iron and zinc. The efficiency of complex formation depends on the chemical properties of the mineral, the pH and the presence of other compounds. Phytate binds preferentially to calcium and iron, limiting their bioavailability but increasing zinc bioavailability. • Organic acids (such as ascorbate and citrate) and cysteine promote iron absorption. EDTA can promote iron and zinc absorption. • Calcium can compete with iron for intestinal absorption; its effect on zinc has not been determined.
Water-soluble vitamins [14,65]
• Vitamins that form complexes in the food matrix are absorbed less efficiently than free vitamins. • Vitamins B1, B3, B6 and B9 can become trapped in the insoluble part of the food matrix in certain plant foods, reducing their bioaccessibility. • Dietary fiber does not have a significant impact on the absorption of water-soluble vitamins.

Table 2. Effect of processing on the micronutrient content of food

Carotenoids [14,26,49,66]	
Drying	Can reduce carotenoid levels but this depends on the drying method, the temperature/time combination and the genotype of the plant source.
Storage	Can cause the loss of carotenoids, but this depends on the crop species, genotype and storage conditions.
Milling	Increases carotenoid bioavailability because the food particle size is reduced.
Blanching	Enhances carotenoid retention because it inactivates peroxidases that can lead to the formation of undesirable colors and flavors, and carotenoids are released from carotenoid–protein complexes.
Fermentation	Does not usually affect carotenoid retention, and can also remove inhibitors and/or favor the accumulation of nutritional promoters.
Nixtamalization	Defined as soaking maize grains in an alkaline solution, which can reduce carotenoid levels. Bioaccessibility depends more on the subsequent processing/cooking method.
Heating	Can increase bioaccessibility by releasing carotenoids from plant tissues and disrupting carotenoid–protein complexes, although this depends on the plant source and the cooking method. Boiling and steaming retain more carotenoids than baking and frying. Exposure to light and long-term heating induces *trans*-to-*cis* isomerization resulting in the loss of provitamin A activity, although photoxidation is the main factor responsible of carotenoid isomerization.
Iron and zinc [32,49,65,67]	
Dehulling	Reduces the level of inhibitors that prevent mineral uptake. This process removes the bran, reducing the amount of fiber and phenolic compounds, but phytate levels still remain high because phytate is also present in the germ. Significant quantities of minerals can also be lost, e.g. up to 50% of the iron in some grains, whereas zinc losses are more variable.
Milling	Degrades the cell wall, allowing minerals to interact with other components. Iron, zinc and phytate levels are reduced by milling, but the remaining iron and zinc is more bioavailable.
Soaking	Can reduce phytate levels by solubilizing phytate or activating endogenous phytases. However, blanching and soaking also cause the leaching of minerals.

Fermentation	Can degrade phytate through the action of microbial phytases. Fermentation can also enhance iron and zinc absorption because low-molecular-weight organic acids are produced during this process. The phytate content is reduced more during fermentation than during cooking.
Nixtamalization	Can reduce iron absorption by competing with calcium, although it may also improve zinc and iron absorption by reducing the phytate content.
Heating	Can enhance mineral absorption by softening the cell walls and removing inhibitors. Minerals are heat-stable, although losses can occur due to leaching. The bioaccessibility of iron is affected more than zinc by the cooking method.
Water-soluble vitamins [14,49,65]	
Drying	Can result in the loss of vitamins, especially air drying which promotes oxidation.
Storage	Does not appear to affect water-soluble vitamins, except B vitamins in long-term storage, and vitamin C due to oxidation during storage.
Dehulling Milling	The grain components are separated, resulting in significant losses of certain B vitamins that accumulate in the bran and germ.
Soaking	Reduces the levels of water-soluble vitamins by leaching.
Blanching	Inactivates enzymes that oxidize vitamin C but also encourages the loss of vitamin C by leaching.
Fermentation	Can increase the bioavailability of certain B vitamins (e.g. B2 and B3).
Nixtamalization	Can reduce the content of certain B vitamins (e.g. B1 and B3), but in some cases the remaining quantity can become more bioavailable (e.g. B3).
Cooking	Can release vitamins from the food matrix but can also destroy heat-sensitive vitamins (B1, B2 and C), although this depends on temperature/time combinations. The greatest losses during cooking occur due to vitamins leaching into the water, so steaming is preferred to boiling.