Chapter

Vitamin B9 in Dark Green Vegetables: Deficiency Disorders, Bio-Availability, and Fortification Issues

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Abstract

Folic acid is a B complex water-soluble vitamin that is essential to humans, and its deficiency can cause problems including neural tube defects as well as heart-related diseases. An important feature of such vitamins is that they are generally not synthesized by mammalian cells and therefore must be supplied in sufficient amounts in the diet. Folate is a generic term for compounds, possessing vitamin activity similar to that of pteroylglutamic acid, and is the form of the vitamin, which is naturally present in foods. The main dietary sources of folic acid are dark green and leafy vegetables such as spinach, asparagus, romaine lettuce, broccoli, bok choy, turnip green, beet, dried or fresh beans, and peas. The amount of folate that is absorbed and utilized physiologically varies among different food sources and different chemical forms of the vitamin. About 85% of folic acid is estimated to be bioavailable; however, the bioavailability of food folate is estimated at about 50% of folic acid. Several national health authorities have introduced mandatory food fortification with synthetic folic acid, which is considered a convenient fortificant, being cost efficient in production, more stable than natural food folate, and superior in terms of bioavailability and bio-efficacy. Presently, many countries affected by diseases associated with a lack of folic acid have made it mandatory to supplement foods with the vitamin. Considering the need, several analytical procedures were standardized to determine the presence of folic acid in different food matrices. The reported methods are simple, selective, robust, and reproducible and can be used in routine analyses.

Keywords: folic acid, vitamin B9, green leafy vegetables, bio-fortification, bio-availabilty

1. Introduction

Vitamin B9 also called folate or folic acid (FA) or pteroyl-L-glutamic acid is one of the eight water-soluble B vitamins. The chemical name of folic acid is *N*-[4-[[(2-amino-3,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]-L-glutamic acid. The chemical formula of folic acid is $C_{19}H_{19}N_7O_6$ and its molecular weight is 441.4. The IUPAC name is (2*S*)-2-[[4-[(2-amino-4-oxo-3*H*-pteridin-6-yl) methyl amino] benzoyl] amino] pentanedioic acid. Folic acid appears as odorless orange-yellow needles. Folic acid is a synthetic form of folate, found in vitamin supplements and fortified foods. Its structure has been shown in **Figure 1**.

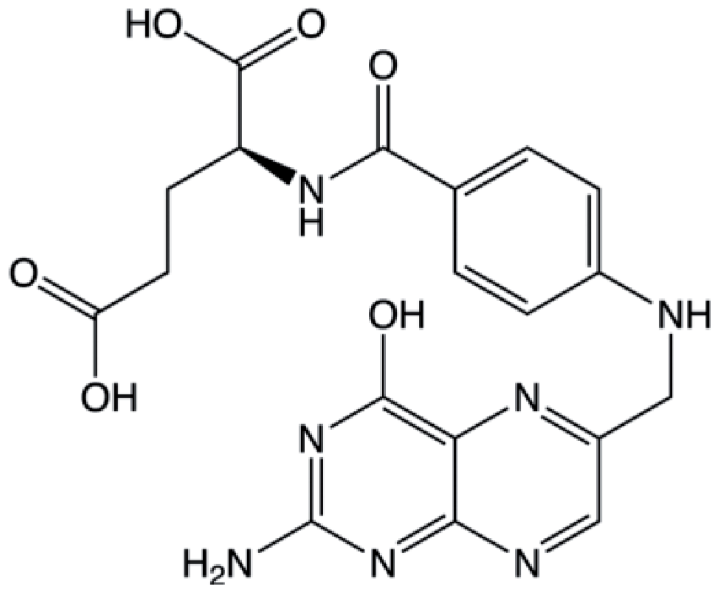

Figure 1.
Chemical structure of the folic acid molecule.

Folate, vitamin B12, and riboflavin have attracted scientific as well as health interest in recent years. Folate has a well-established role in preventing neural tube defects (NTDs); however, there are several other reports highlighting the potential role of folate and B-vitamins in protecting against several lifestyle diseases including heart-related cardiovascular diseases (especially strokes), certain types of cancers, cognitive impairment, and bone-related osteoporosis. Folic acid is involved in carbon transfer reactions of amino acid metabolism, in addition to purine and pyrimidine synthesis, and is essential for hematopoiesis and red blood cell production. It is found in many foods and particularly in leafy green vegetables that are essential for the critical biosynthetic pathways involving the transfer of methyl groups to organic compounds. Folate is important for a range of functions in the body. Folic acid (vitamin B9) works with vitamin B12 and vitamin C to help the body break down, use, and make new proteins. Folate is required in the synthesis of nucleic acids *viz*., DNA and RNA and is also part of the protein metabolism. It helps in the degradation of homocysteine, which is a risk factor for heart disease. Folic acid is required for growth, reproduction (during gestation and lactation), and antibody formation. As a coenzyme, it is involved in glycine metabolism and is essential for the synthesis of purines, as well as pyrimidines. It plays a major role in cell division and protein synthesis. Its deficiency induces chromosomal abnormalities [1]. It is essential for the formation of RBCs and prevention of folate deficiency anemia [2]. Folic acid deficiency can lead to congenital malformations in the fetus (spina bifida, encephalocele, cleft palate, and hydrocephalus), as well as heart disease [3–5]. Deficiency of folate leads to megaloblastic anemia (a condition where there is a reduction in RBCs, and the red blood cells are larger in size than normal). Other symptoms include weakness, fatigue; irregular heartbeat, shortness of breath, hair loss, pale skin, mouth sores, etc. The nutrient is very important during early pregnancy to reduce the risk of birth defects of the brain and spine [1]. An important feature of this vitamin is that they are generally not synthesized by mammalian cells and therefore must be supplied in sufficient amounts in the diet [6, 7].

2. Recommended dietary allowance (RDA)

The RDA for males and females aged 15 years and older is 400 μg DFE day^{-1} (**Table 1**), whereas the RDA ranges from 65 to 300 μg DFE day^{-1} for ages between

Category	Age	RDA (μg day^{-1})
Infants	0–6 months	65
	6–12 months	80
	1–3 years	150
Children	4–6 years	200
	7–14 years	300
Adults	15+ years	400
Pregnancy		600
Lactation		500

Source: Miller [8].

Table 1.
Recommended dietary allowances (RDA) for folate.

0 and 14 years. The requirement of folate for pregnant and lactating women is comparatively higher, respectively, 600 and 500 μg DFE day^{-1}. The upper tolerable limit (UL) for folic acid has been reported as 1000 μg day^{-1}. Men and women of age 19 years and older should aim for 400 μg DFE. Those who are breastfeeding should aim to take around 500 μg per day. People who regularly drink alcohol should aim for at least 600 mcg DFE of folate daily since alcohol can impair its absorption. Higher daily doses (up to 4 mg) are recommended for women who have had a baby with a neural tube defect. Folate is essentially nontoxic, although there is some concern that high doses may mask pernicious anemia. The body absorbs folic acid from supplements and fortified foods better than the folate from naturally occurring foods.

3. Common symptoms of folate deficiency

A diet lacking folate or folic acid can lead to a folate deficiency. Inadequate levels of folate (vitamin B9) and vitamin B12 during pregnancy have been reported to lead to an increased risk of neural tube defects (NTDs) [9]. Although both are part of the same biopathway, folate deficiency is much more common and therefore it is of much concern [10]. NTDs are birth defects of the brain, spine, or spinal cord and Spina bifida as well as anencephaly are the most common ones. The spinal column of the fetus does not close completely in spina bifida, whereas in anencephaly, most of the brain and skull do not develop properly due to which babies with anencephaly are either stillborn or may die shortly after birth. The peri-conceptional folate supplementation can reduce the risk of neural tube defects (NTDs) and other congenital abnormalities such as cardiovascular malformations (CVMs), cleft lip and palate [4], urogenital abnormalities, and limb reductions [11]. Supplementation with folic acid reduces the prevalence of NTDs by approximately 70% indicating that 30% of these defects are not folate-dependent and are due to some other reasons, rather than alterations of methylation patterns. Many other genes related to NTDs exist, which may be responsible for folate insensitive NTDs. However, folate deficiency can also occur in people who is suffering from celiac disease that prevents the small intestine from absorbing nutrients from foods (malabsorption syndromes). Deficiency of folic acid can cause a wide range of problems in the human body, which may include tiredness, fatigue, and lethargy, besides muscle weakness and other neurological signs, such as tingling, burning, or peripheral neuropathy leading to numbness. It may also cause psychological problems, such as depression and memory problems, and gastrointestinal symptoms, such as nausea, vomiting, abdominal pain, weight loss and diarrhea, headache and dizziness, and shortness of breath. Anemia, particularly megaloblastic anemia, is often the first sign that there is an underlying folate deficiency, and doctors will usually test for folate deficiencies when they encounter anemia. In pernicious anemia, our immune system attacks healthy cells in our stomach, which prevents the absorption of vitamin B12 from the food we eat, and this is the most common cause of vitamin B12 deficiency. Other factors include the lack of these vitamins in our diet. Besides this, certain medicines, including anticonvulsants and proton pump inhibitors (PPIs), can affect how much of these vitamins our body absorbs both. Vitamin B12 deficiency and folate deficiency are more common in older people. The folates are hydrolyzed to monoglutamate in the gut before absorption by active transport across the intestinal mucosa. Sometimes, passive diffusion also occurs when pharmacological doses of folic acid are consumed. Before it enters the bloodstream, the enzyme dihydrofolate reductase reduces the monoglutamate to tetrahydrofolate [12]. The major folate in plasma is 5-methyl-THF. The activity of dihydrofolate reductase varies among individuals [13]. It is yet not known whether the unmetabolized folic acid has any biological activity or it can be used as a biomarker of folate status [14]. Folate is also synthesized by colonic microbiota and can be absorbed across the colon, although the extent to which colonic folate contributes to folate status is unclear [15]. The folate content of the body is estimated to be around 15–30 mg. Half of this amount is stored in the liver and the rest amount is found in blood and body tissues [16]. Normally, the serum folate concentration is used to assess the folate status of the body. A value higher than 3 ng/mL indicates adequacy [17]. The erythrocyte folate concentration provides a longer-term measure of folate intake; a concentration above 140 ng/mL indicates adequate folate status [13, 17]. A combination of serum or erythrocyte folate concentration can also be utilized to assess folate status. Sometimes plasma homocysteine concentration is also used as a functional indicator of folate status because homocysteine levels rise when the body is unable to convert homocysteine to methionine due to deficiency of 5-methyl tetra hydrafolate [17]. Homocysteine levels, however, are not a highly specific indicator of folate status because they can be influenced by other factors, including kidney

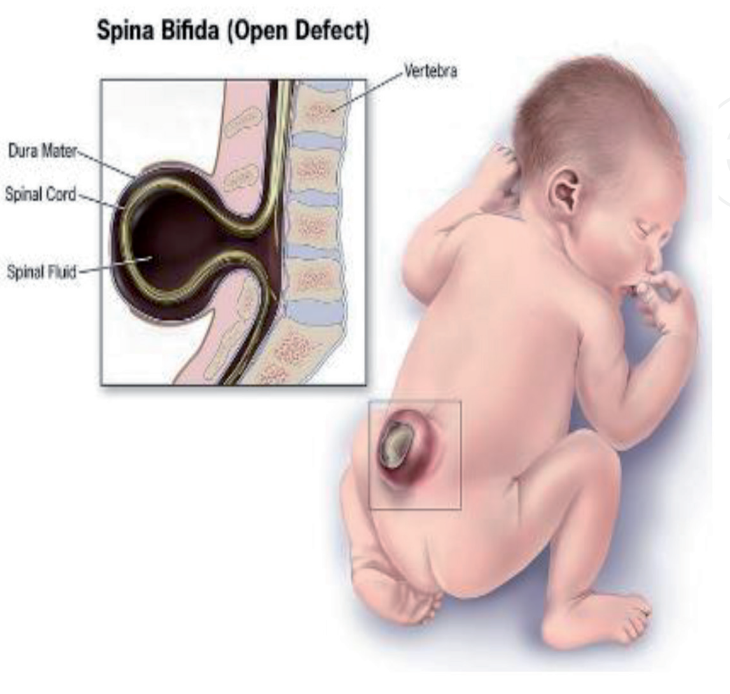

Figure 2.
Neural tube defect—Spina bifida.

dysfunction and deficiencies of vitamin B12 and other micronutrients [17, 18]. The most commonly used cutoff value for elevated homocysteine levels is 16 μmol/L. A homocysteine cutoff of 10 μmol/L has been proposed for assessing folate status in populations (**Figure 2**) [13].

4. Food containing folic acid

Folic acid is not found in natural food sources; however, folate is the natural form of vitamin B9, which is water soluble and naturally found in many foods commonly in dark green and leafy vegetables such as spinach, asparagus, romaine lettuces, broccoli, bok choy, turnip green, beet, dried or fresh beans, and peas (**Table 2**). The main dietary sources of folate are spinach, white beans, asparagus, dark-green leafy vegetables, Brussels sprouts, soybean, orange, and melons [19]. In addition to the aforesaid, beef liver, black-eyed peas, asparagus, lettuce, avocado, broccoli, mustard greens, green peas, kidney beans, canned tomato juice, orange juice, dry-roasted peanuts, fresh orange and grapefruit, papaya, banana, hard-boiled egg, and cantaloupe are also good sources of folate. Other sources of this vitamin include sunflower seeds, avocados, peanuts, orange juice, pineapple juice, cantaloupe, honeydew melon, grapefruit juice, banana, raspberry, papaya, grapefruit, strawberry, corn, and wheat germ. Among animal products, liver (the folate storage organ in mammals) and liver products, whole eggs, and baker's yeast are rich in folates. The major staple crops of the globe *viz*., rice and maize are low in folates (**Table 3**) [20]. However, pulses and other legumes and green

Sr. No.	Vegetables	Folate content (μg/100 g)
1	Broccoli raw	63
2	Brussels sprout, raw	61
3	Kale, raw	141
4	Collards, raw	129
5	Endive, raw	142
6	Cauliflower green, raw	57
7	Cabbage Chinese (pak-choi), raw	66
8	Cabbage Chinese (pe-tsai), raw	79
9	Cabbage savoy, raw	80
10	Cabbage, raw	43
11	Cauliflower green, raw	57
12	Parsley, fresh	152
13	Spinach, raw	194
14	Peas, edible podded, raw	42
15	Peas green, raw	65
16	Soybean green, raw	165
17	Cowpea (black eyes) immature seeds, raw	168
18	Fava Bean pods, raw	148

Source: USDA Nutrient Database for Standard Reference.

Table 2.
Folate content in raw vegetables.

Sr. No.	Cereals	Folate content (μg/100 g)
1	Rice, brown, long grain, raw	23
2	Rice, white, short grain, raw	06
3	Wheat flour, whole grain, soft wheat	28
4	Wheat flour, whole grain	44
5	Wheat, durum	43
6	Corn grain, yellow	19
Sr. No.	**Pulses**	**Folate content (μg/100 g)**
1	Adzuki bean	622
2	Black bean	444
3	French bean	399
4	Kidney bean red	394
5	Chickpea	557
6	Lentil	479
7	Mung bean	625
8	Peas, split	274
9	Cowpea	659
10	Pigeon pea, immature seeds	173

Source: USDA Nutrient Database for Standard Reference.

Table 3.
Folate content in raw cereal grains and legume seeds.

Food	Serving size	Folic acid/folate per serving* (μg)
Asparagus (cooked)	4 spears	88
Avocados	1 ounce	19
Beans		
Black Beans (cooked from dried)	1 cup	256
Kidney/red beans (canned)	1 cup	131
Lentils (cooked from dried)	1 cup	358
Pinto beans (cooked from dried)	1 cup	294
Chickpeas/Garbanzo beans (canned)	1 cup	161
Chickpeas/garbanzo beans(cooked from dried)	1 cup	282
Blackeye peas (canned)	1 cup	122
Blackeye peas (cooked from dried)	1 cup	358
Bread products (made with enriched flour)		
Bread, white	1 slice	35
Bread, whole wheat	1 slice	14–26
Bread, bagel	1–4 inch bagel	119
Broccoli (cooked)	1 cup	78
Collard greens (cooked)	1 cup	177
Corn on the cob (cooked)	1 ear	35

Food	Serving size	Folic acid/folate per serving* (μg)
Corn (frozen and cooked)	1 cup	51
Okra (cooked)	1 cup	74
Orange	1 orange	39
Orange juice	1 cup	110
Papaya	1 cup	53
Peas		
Green peas, cooked (frozen or canned)	1 cup	75–94
Split peas (cooked from dried)	1 cup	127
Rice, enriched (cooked)	1 cup	195–222
Soybeans (cooked)	1 cup	93
Spinach (raw)	1 cup	58
Spinach (cooked)	1 cup	263

Source: USDA Nutrient Database for Standard Reference, Release 15.

Table 4.
Folate content in cooked food.

leafy vegetables such as spinach, asparagus, lettuce, and Brussels sprouts are rich in folates (**Tables 2** and **3**). It has been reported that folate concentration in rice cultivars ranged from 11.0 to 51 μg/100 g with a mean of 26.0 μg/100 g [20, 21]. Singh [22] have also reported that legumes are a rich source of folates, followed by green vegetables, spices, and cereals. According to USDA [23], the amount of folates varies from 0.1 to 0.5 μg/g in brown rice, which is reduced by 60–67% during milling of rice. The folate content deteriorates on long storage (23%) and also during boiling (48.3%). Folate intake is strongly influenced by various methods of cooking that can degrade the natural forms of the vitamin in foods (**Table 4**). Steaming of spinach or broccoli, in contrast, resulted in no significant decrease in folate content, even for the maximum steaming periods of 4.5 min (spinach) and 15.0 min (broccoli).

5. Folate bioavailability

Folates from natural food sources can enhance the folate status only to a limited extent because of their poor stability while being cooked and also less bioavailability when compared with the synthetic vitamin and folic acid [24]. In addition to the less bioavailability of food folates, the poor stability of folates in foods (particularly green vegetables) while cooking can substantially reduce the amount of vitamin, which is ingested, and this may be an additional factor that limits the ability of food folates from naturally available cooked foods to enhance the folate status. Folate bioavailability from different foods is considered to be dependent on several factors, including the food matrix, the intestinal deconjugation of polyglutamyl folates, the instability of certain labile folates during digestion, and the presence of certain dietary constituents that may enhance folate stability during digestion. However, limited folate bioavailability data are available for vegetables, fruits, cereal products, and fortified foods; hence, it is difficult to evaluate the bioavailability of food folate or whether intervention with food folate improves folate status. The amount of folate that is absorbed and utilized physiologically varies among different food sources and different chemical forms of the vitamin. At least 85% of folic acid is estimated to be bioavailable when taken with food [12, 25], whereas the bioavailability of food folate is commonly estimated at about 50% of folic acid bioavailability [26], but this should be considered as a rough estimate, as data on bioavailability of food folate vary between 30 [24] and 98% [27]. The chemically most stable folate form is synthetic folic acid [28], which is cheap to produce and therefore used for dietary supplements and food fortification. The folic acid consumed as a supplement is highly bioavailable.

It has been reported that the polyglutamyl form of food folates is absorbed in the jejunum as monoglutamyl folate after removal of the polyglutamyl chain by intestinal γ-glutamyl hydrolase [29], which is thereafter reduced and methylated in the enterocyte. The extent of passive diffusion of the reduced and methylated folate across the cell membrane is very limited [30], as it takes place only at high doses. To some extent folate is also absorbed in the colon, and it is suggested that colonic absorption may contribute significantly to total folate absorption [31], but it is still unknown that how relevant this absorption is for maintaining folate status. However, it has been shown for humans [32] that folates synthesized by colon bacteria are bioavailable. Absorbed folate is transported to the liver, which contains about half the body pool of folate [33] and retains 10–20% of absorbed folate due to the first-pass effect [34], while the rest is transported *via* the systemic circulation to body tissues. Some liver folate participates in the enterohepatic circulation and is secreted into bile [35]. However, most biliary folate is reabsorbed, supposedly to moderate between-meal fluctuations in folate supply to cells [36].

National Health and Nutrition Examination Survey data (NHANES 2013–2014) show that the majority population in the United States consume adequate amounts of folate. The average daily intakes of folate from foods range between 417 and 547 μg DFE per day for children between 2 and 19 years of age [37]. However, the mean dietary intakes for males who are 20 years and older are 602 μg DFE and for females, it is 455 μg DFE. It has been reported that although most of the people in the United States consume adequate amounts of folate, there are certain groups such as women of childbearing age and non-Hispanic black women who are still at risk of insufficient folate intakes. It has been further reported that about 35% of adults and 28% of children aged 1–13 years in the United States use supplements containing folic acid [38, 39] to meet their folate requirement. According to estimates of USDA-ARS [37], people aged 2 years and older who consume supplements containing folic acid get a mean of 712 μg DFE from those supplements. Several studies suggest that measurements of folate levels in the erythrocytes further confirm that most people in the United States have adequate folate status. Further there are also some analyses (NHANES 2003–2006), which shows that less than 0.5% of children (aged 1 to 18 years) have deficient folate concentrations in the erythrocytes [40]. Mean concentrations in this age group range from 211 to 294 ng/mL depending on age, dietary habits, and the amount of supplement use. In adults, mean erythrocyte folate concentrations range from 216 to 398 ng/mL, which also indicates the adequate folate status [39].

In contrast to this, there are also reports that some of the population groups are at risk of obtaining excess folic acid, primarily because of the folic acid they obtain from dietary supplements. About 5% of men and women aged between 51 and 70 years and men aged 71 years and older have folic acid intakes exceeding the prescribed upper limit of 1000 μg per day [38]. Furthermore, 30–66% of children aged 1–13 years who take folic acid-containing supplements have intakes of folic acid from both fortified food and dietary supplements exceeding the upper limit of 300–600 μg per day [39]. Almost all children aged 1 to 8 years who consume at least 200 μg/day folic acids from dietary supplements have total intakes that exceed the upper limit [40]. Despite so many reports of excess intakes of folic acid, there is very little information available about the long-term effects of consumption of high folic acid doses in children [14].

6. Folic acid biofortification

Biofortification is a promising and sustainable agriculture-based strategy to minimize Zn and Fe deficiency in dietary food substances [41]. Among the different strategies deployed, the plant breeding approach to develop biofortified crops and agronomic supplementation of micronutrients, such as foliar/soil application along with chemical fertilizers, have received maximum attention [42]. Breeding staple food crops for higher micronutrient contents, where the density of minerals and vitamins in food staples may be increased either through conventional plant breeding or using transgenic techniques. It is recognized as a nutrition-sensitive-agriculture intervention that can reduce vitamin and mineral deficiency [43]. Iron biofortification of beans, cowpea and pearl millet, zinc-biofortification of maize, rice, and wheat, and pro-vitamin A carotenoid-biofortification of cassava, maize, rice, and sweet potato are currently underway and at different stages of development [44, 45]. Results are promising for iron-biofortified crops, as partially iron-biofortified rice has improved the iron stores of reproductive-age women in the Philippines [46], iron-biofortified pearl millet has increased the iron stores and reversed iron deficiency in school children in India [47], and iron-biofortified beans have improved the iron stores in women in Rwanda [48]. The agronomic mode of biofortification includes the application of micronutrient fertilizer directly to the soil and/or foliar application. The agronomic biofortification is most suitable for staple crops with starch as the major component and is mainly practiced on crops such as rice, wheat, maize, sorghum, millet, and sweet potato and also on legumes. The foliar fertilization often results in more uptake of nutrients and ultimately efficient allocation in the edible plant parts [49]. Soil and foliar application combined together gives better results and has been shown as the most effective method for biofortification [42, 50]. Foliar application of micronutrients is generally much more effective in ensuring uptake into the plant because in such cases, immobilization of the nutrients in the soil can be avoided. Alternatively, microorganisms have been bioengineered to overproduce folates. *Bacillus subtilis* was modified at three different levels and an eightfold increase in folate levels was observed [51]. Metabolic engineering has also been developed in *Lactococcus lactis* leading to a more than threefold increase [52].

Several national health authorities have introduced mandatory food fortification with synthetic folic acid, which is considered as convenient fortificant, being cost efficient in production, more stable than natural food folate, and superior in terms of bioavailability and bio-efficacy. It has been reported that the mandatory folic acid fortification in such countries leads to significant increase in both serum and erythrocyte folate concentrations in all sex and age groups. Studies have shown that the mean serum folate concentration increased more than twofold (136%) and the mean erythrocyte folate concentration increased by 57%. The introduction of folic acid-fortified staple foods has effectively decreased the prevalence of NTD in the United States and Canada [53]. It was also observed that fortifying flour with iron and many water-soluble B group vitamins in the United States, Canada, and many other countries has resulted in preventing micronutrient deficiency conditions and is also a very cost-effective prevention of major neural tube defects *viz*., spina bifida and anencephaly, and also folate deficiency anemia. There are also reports showing that fortification with folic acid has led to a reduction in cases of heart diseases like strokes, which occur due to elevated homocysteine levels. According to the published reports, around 50 countries have implemented the mandatory folic acid fortification program, including the United States and Australia.

In January 1998, the U.S. Food and Drug Administration (FDA) suggested the processing industries to add 140 μg folic acid/100 g to enriched bread, cereals, flours, corn meals, pasta, rice, and other grain products [54] to reduce NTDs. Because cereals and grains are used as staples and are widely consumed, these products have become important supplement of folic acid to the diet. The fortification program increased mean folic acid intakes in the United States by about 190 μg/day [54]. Many other countries, including Costa Rica, Chile, and South Africa, have also established mandatory folic acid fortification programs [55, 56].

7. Analytical methods for the determination of folic acid

Methods reported in the literature for the determination of folic acid include HPLC with different detectors [57], electrophoresis [2], electrochemical methods [58], flow injection analysis [59], and spectrophotometric methods. Recently, some novel spectroscopic methods were reported for routine determination of folic acid. These methods are based on the formation of colored species on binding of folic acid with sodium nitroprusside and ammonia reagent to produce a dark yellow-colored chromogen (λmax at 390). A new, simple, easy, accurate, precise, economic, and sensitive UV spectrophotometric method for the determination of folic acid in commercial tablets has been reported. It was possible to determine the concentration of folic acid in commercial tablets at a λmax of 282.5 nm in a linear range of 1.0–17.5 μg mL^{-1} with an $R2 > 0.9999$ and recovery between 100.6 and 101.1% using a phosphate buffer solution at pH 9.0. De Moura Ribeiro Vinicus et al. [60] reported the spectrophotometric methods for the determination of folic acid in different pharmaceutical formulations, using 0.1 mol L^{-1} NaOH as solvent. This method is simple, selective, and robust.

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