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Irradiation Stability of Folic Acid in Powder and Aqueous Solution

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ABSTRACT: This study attempts to examine the folic acid stability after irradiation treatment, under different physical states, pH values, and atmosphere conditions. Aqueous folic acid samples, folic acid in powder, and wheat flour fortified with folic acid were irradiated by an electron beam (E-beam) between 0 (control) and 10.0 kGy. It was realized that the physical state of folic acid plays an important role on its stability toward E-beam processing, being largely unstable in solution, no matter the pH and atmosphere conditions assayed. Otherwise, folic acid in powder showed huge irradiation stability, even when mixed in a dry food matrix, such as fortified wheat flour samples.

KEYWORDS: Radiation processing, folic acid, stability, wheat flour

INTRODUCTION

Folates are a group of vitamins based on the parent compound folic acid (FA) [pteroylglutamic acid (PGA)]. They belong to the B vitamin group and are an essential food element in the human diet. FA is a yellowish orange crystalline powder that is tasteless and odorless. It is composed of a pteridine ring, *p*-amino benzoic acid (PABA), and glutamate moieties (Figure 1). Separately, the three moieties have no vitamin activity.[1]

The human metabolism is not able to synthesize folates and, therefore, has to obtain them from the diet. Dietary sources rich in folates include yeast extracts, liver, eggs, kidney, green leafy vegetables, legumes, and citrus fruits.[2] A deficiency of folate in the diet is closely linked to the presence of neural tube defects in newborns and an increased risk of megaloblastic anemia, cancer, Alzheimer's disease, and cardiovascular disease in adults.[3,4] This has led to the fortification of some foodstuffs with FA.[5] FA, a synthetic vitamer, is used as a food fortificant in cereals and dairy products because of its low price, relative stability, and increased bioavailability compared to natural folate forms.[6,7] Following other countries, in 2002, the Brazilian Agency for Public Health Surveillance (ANVISA) required the mandatory fortification of wheat and corn flours with FA at a concentration of 1.50 μg g^{-1} of flour.[8]

New food product development and food processing aims to retain as much as possible of the naturally occurring vitamin content, to protect added vitamins, and to minimize the appearance of undesirable neo-formed products.[9] The stability of folates during food processing is influenced by temperature, pressure, pH, oxygen, light, metal ions, antioxidants, and duration of heating.[10−12] More and more, industries are using "non-thermal technologies" to treat food products. Among the so-called "non-thermal technologies", food irradiation is a process in which food is exposed to ionizing radiations, such as high energy electrons and X-rays produced by electron accelerators [electron beams (E-beams)] or γ rays emitted from the radioisotopes ^{60}Co. Dependent upon the absorbed radiation dose, various effects can be achieved resulting in reduced storage losses, extended shelf life, and/or improved microbiological and parasitological safety of foods.[13] Food irradiation is one of the most effective means to disinfect dry food ingredients. Disinfestation is aimed at preventing losses caused by insects in stored grains, pulses, flour, cereals, coffee beans, dried fruits, dried nuts, and other food products.[14] The dosage required for insect control is fairly low, of the order of 1 kGy or less.[15] In comparison to γ ray installations, E-beam facilities do not need reloading and can be streamlined to food processing, reducing logistic costs.[16] Safety and efficiency of food irradiation has been proven by several authorities [Food and Drug Administration (FDA), United States Department of Agriculture (USDA), World Health Organization (WHO), Food and Agriculture Organization (FAO), etc.] and scientific societies based on extensive research.[17,18]

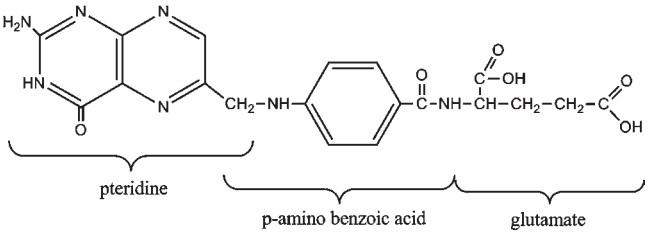

Figure 1. Structural formula of FA at pH 7.

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Previous authors have already studied the effect of radiation processing in natural folates and FA in foods[19,20] and the thermal/photostability of the synthetic folate form, FA.[21−23] The novelty of this work was to examine the effect of irradiation on the stability of FA under different moisture contents, atmosphere conditions, and pH values. Indeed, because FA is unstable under acid medium, alkaline and neutral pH values were analyzed. A neutral solution of FA was allowed by the addition of ammonium acetate to water FA suspensions. The irradiation effect was analyzed on not only the synthetic vitamer itself but also the FA-fortified wheat flour.

MATERIALS AND METHODS

Materials. *Chemicals.* Methanol (MeOH) (Carlo Erba, Val de Reuil, France) and acetonitrile (ACN) (Sigma-Aldrich, Steinheim, Germany) were high-performance liquid chromatography (HPLC)-grade. Methanol for Karl Fisher determination (Carlo Erba, Val de Reuil, France) was European-Pharmacopeia-grade. All other chemicals were of analytical grade. Formic acid was purchased from Riedel-de Haen (Seelze, Germany). Sodium hydroxide (NaOH) was purchased from SDS (Peypin, France). Ammonium acetate, dibasic sodium phosphate, and monobasic potassium phosphate were purchased from Merck (Darmstadt, Germany). FA was obtained from Sigma-Aldrich (Steinheim, Germany). Water was purified using a Synergy Milli-Q System (Millipore, Molsheim, France).

Samples. *FA.* All FA aqueous solutions and suspensions were prepared under subdued light, to protect folates from oxidative degradation induced by light. To assess both the pH-dependent response and solubilization effect, FA in powder, solutions, and suspensions were prepared under four different conditions: (a) A total of 100 mg of FA was added to 100 mL of water and solubilized by the addition of 5.0 mL of 1 M NaOH. The solution was diluted in water to a final concentration of 0.1 mg/mL, resulting in a pH of 12. (b) A total of 100 mg of FA was added to 100 mL of water and solubilized by the addition of 0.5 mL of 1 M NaOH. The solution was diluted in water to a final concentration of 0.1 mg/mL, resulting in a pH of 8. (c) A total of 100 mg of FA was added to 1000 mL of water and solubilized by the addition of 100 mL of 0.4 M ammonium acetate, resulting in a final concentration of 0.1 mg/mL at a pH of 6.8. (d) A total of 70 mg FA was added to 700 mL of water, resulting in a final concentration of 0.1 mg/mL (suspension). (e) FA was in powder.

Prior to irradiation, a set of samples were sealed in the presence of oxygen and another set of samples were sealed in the presence of nitrogen (obtained by flushing the air gap with pure nitrogen gas for 1 min).

All samples (4 mm thick) were packed in plastic sachets, sealed, and labeled with their respective radiation doses.

Folate solutions were directly injected to the chromatographic system. Folate suspensions were first solubilized by the addition of 1 M NaOH (5 μL to each 1 mL of 0.1 mg/mL FA), prior to injection. FA in powder was first added to water at 1 mg/mL and solubilized by the addition of 1 M NaOH (50 μL to each 1 mL of 1 mg/mL solution). Prior to injection, this solution was diluted in water to a final concentration of 0.1 mg/mL.

Wheat Flour Fortified with FA. Brazilian wheat flour fortified with FA were purchased in different local supermarkets in São Paulo (Brazil) in July 2010 (positive control). Non-fortified wheat flours were purchased in local supermarkets in Strasbourg (France) in July 2010 and fortified in house with FA at a final concentration of 1.50 μg g^{-1}. Blending was carried out with a Turbula T2F shaker (Wab, Basel, Switzerland) at a speed of 72 rpm and a fill volume of approximately 25%. First, 45 mg of FA was blended with 300 g of wheat flour for 2 min, resulting in a final concentration of 150 μg g^{-1}. Second, 30 g (150 μg g^{-1} mixture) was blended with 270 g of wheat flour for 5 min, resulting in a final concentration of 15 μg g^{-1}. Finally, 3 g (15 μg g^{-1} mixture) was blended with 297 g of wheat flour for 10 min, resulting in a final concentration of 1.5 μg g^{-1}.

A total of 20 g of samples (4 mm thick) were packed in plastic sachets, sealed, and labeled with their respective radiation doses.

Methods. *Irradiation Treatment.* FA powder samples were irradiated with a Rhodotron E-beam accelerator (IBA, Louvain la Neuve, Belgique), with a 15 mA current, 160 cm scan width, and 10 MeV energy. Applied doses (maximum of ±10%) were 0 (control), 1.0, 3.0, 5.0, 7.0, and 10.0 kGy.

FA aqueous solutions and suspensions and wheat flours fortified with FA were irradiated with a Van de Graaff E-beam accelerator, 2 MeV (Vivirad High Voltage, Handschuheim, France) with a 100 μA current, 20 cm scan width, and about 2 kGy/s dose rate. Applied doses were 0 (control), 0.3, 0.54, 0.79, 1.0, 3.4, 5.3, 7.5, and 10.7 kGy. Surface-absorbed doses were monitored with FWT 60.00 radiochromic dosimeters (Far West Technology, Goleta, CA), previously calibrated with an alanine dosimeter (Aérial, Illkirch, France).[24] Dose uniformity of about 10% within the sample was achieved by the use of a 100 μm thick copper scattering foil.[25] For both irradiation plants, standard conditions for temperature and pressure were used (25 °C and 1 atm).

Extraction. A total of 2 g of wheat flour sample was extracted once with 10 mL of extraction phosphate buffer at pH 6.3 (0.25 M Na_2HPO_4 and 0.37 M KH_2PO_4), adapted from Alaburda et al.[26] The mixture was shaken for 30 min in a rotational shaker and centrifuged at 3500 rpm for 15 min. The supernatant was filtered through a 0.45 μm hydrophilic polyvinylidene fluoride (PVDF) membrane (Millipore, Carrigtwohill, Ireland) before chromatography analysis.

HPLC. Experimental work was performed using a Varian ProStar HPLC system (Palo Alto, CA) [9012 binary HPLC pump, 9300 injector, and 330 diode array detection (DAD) UV−vis detector]. Chromatographic separation was carried out on a Hypersil Gold aQ C18 analytical column (4.6 × 250 mm; 5 μm particle size) (Thermo Scientific), protected by an ODS precolumn (Uniguard, Thermo Scientific).

For FA preparations (100 ppm), the mobile phase consisted of aqueous formic acid (pH 3.5) and methanol, at a flow rate of 1.0 mL/min. Gradient elution started at 5% MeOH maintained for 10 min, followed by raising the MeOH concentration linearly to 50% within 20 min. Subsequently, the MeOH concentration decreased again to 5% in 1 min, and the column was equilibrated for 14 min with the initial mixture. An injection volume of 20 μL was used.

For wheat flour preparations (1.5 ppm), a gradient elution with acetonitrile and aqueous acetic acid (pH 2.8) was used[26] to obtain a better chromatographic resolution and a subsequently higher sensitivity of detection. The flow rate was 0.7 mL/min. The gradient started at 8% ACN, and after 10 min, the ACN proportion was linearly raised to 26% within 1 min. It was isocratically maintained for 5 min. Thereafter, the ACN concentration was linearly raised to 50% within 6 min and isocratically maintained for 9 min. The ACN proportion came back to 8% within 5 min, and the column was restabilized for 10 min before the next injection. An injection volume of 20 μL was used.

Both detections were performed at 280 nm. To control the analytical system, a negative control (H_2O) and a positive control (0.1 mg/mL FA aqueous solution at pH 12) were injected throughout the sample analysis every 10 injections. FA identity was controlled by its UV spectra (DAD).

Karl Fischer Titration. Titration was performed using a TitroMatic KF 1S-2B titrator (Crison, Barcelona, Spain). A total of 2 g of wheat flour was added to methanol (final volume of 50 mL) and agitated for 15 min at 50 °C. After cooling to room temperature and sedimentation, the supernatant (3 mL) was used for titration for 5 min. The blank sample was made of pure methanol.

Statistical Analysis. Data were compared by analysis of variation (ANOVA) and Tukey's test (at a significance level of 95%) using Statgraphics Plus software. All samples were analyzed in triplicate.

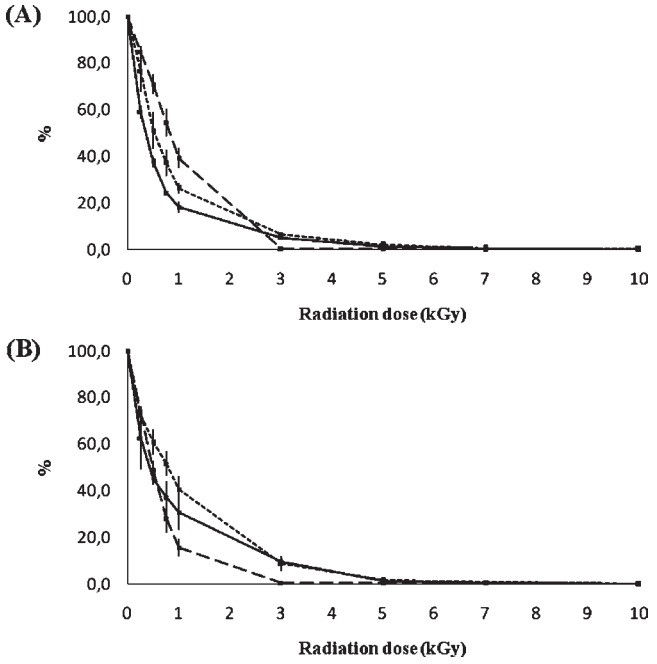

Figure 2. Recovery percentage of FA in aqueous solution under different pH values with (A) O_2 and (B) N_2 (—, pH 12; · · ·, pH 8; - - -, pH 6.8).

RESULTS AND DISCUSSION

Irradiation Effect on FA Solutions. All of the FA aqueous solutions showed a decreasing relationship between their concentration and the absorbed radiation doses (Figure 2). The effects of irradiation on water are extremely important, and they have already been extensively discussed in the literature. Water forms a number of radiolytic products, e.g., hydroxyl radicals (·OH), hydrated electrons ($e^-_{H_2O}$ or e^-_{aq}), hydrogen atoms (·H), hydrogen molecules (H_2), hydrogen peroxide (H_2O_2), and hydrated protons (H_3O^+), each of which may react with compounds and food components.[14] The hydroxyl radical is a powerful oxidizing agent, while the hydrated electron is a strong reducing agent. As a result, both oxidation and reduction reactions take place when compounds and/or foods containing water are irradiated.[14,27] To assess the irradiation effect in these solutions, similar to toxicological and radiological studies, a proposed D_{50}, the half maximal degradation dose, was calculated for each condition assayed. This value intends to represent the radiation dose required for a 50% degradation of FA. FA aqueous solutions showed a marked degradation even at doses below 1 kGy (Figure 2). There is an expressive reduction in the FA percentage, regardless of the pH and/or atmosphere conditions assayed. For FA aqueous solutions at pH 12, D_{50} values of 0.37 and 0.5 kGy were found with O_2 and N_2, respectively (Table 1). Nearby D_{50} values (0.53 and 0.75 kGy) were found for FA aqueous solutions irradiated at pH 8 in the presence of O_2 and N_2, respectively (Table 1). For each radiation dose applied (0.25−10 kGy), no statistical differences were found between FA aqueous solutions at pH 12 (with O_2 and N_2) as well as between FA aqueous solutions at pH 8 (with O_2 and N_2) ($p > 0.05$) (Figure 2). The absence of the difference between solutions with O_2 and N_2 could be explained because, in equilibrium with air, water contains small amounts of dissolved oxygen, which can be reduced by hydrogen atoms to the hydroperoxy radical (·HO_2), a mild oxidizing agent. The hydroperoxy radical is in equilibrium with the superoxide radical (·O_2^-), and both of them can produce hydrogen peroxide in a reaction that consumes oxygen; therefore, an anaerobic matrix may be produced by electron irradiation at dose rates as high as such encountered during E-beam processing.[14] Contrary to these results, Thomas et al.,[29] showed that FA in acid aqueous solutions is photostable in the absence of oxygen when compared to the same samples in the presence of oxygen. On the other hand, for FA aqueous solutions at pH 6.8, D_{50} values were 0.8 and 0.42 kGy with O_2 and N_2, respectively (Table 1). For these samples, the atmosphere condition (O_2 or N_2) showed a significant difference among 0.5, 0.75, and 1 kGy irradiated samples ($p < 0.05$). With doses higher than 5 kGy, for all solutions, a complete FA degradation was reached ($p > 0.05$). Previous authors who studied the irradiation effect in FA stability did not perform assays under different media. On the other hand, several authors studied the effect of FA photolysis under different pH values. Akhtar et al.[22] proposed that the photolysis rate of FA gradually decreased upon moving from acid to alkaline pH values. However, from our results, samples solubilized in ammonium acetate (pH 6.8) appeared to be as susceptible to irradiation as samples solubilized in NaOH (pH 8 and 12) (Figure 2). Surprisingly, ammonium-acetate-solubilized samples showed an increased radiation effect in the absence of oxygen when compared to the same samples in the presence of oxygen. Indeed, samples solubilized in ammonium acetate (pH 6.8, with O_2 and N_2) showed a statistical difference compared to FA aqueous solutions at pH 8 and 12. Therefore, once solubilized, FA is much more unstable to irradiation treatment compared to photolysis.[22,28] This fact could be due to the energy involved in E-beam treatment, which is an in-depth treatment and, in comparison to photolysis, is a much more energetic process and has a higher dose rate. It is already known that photolysis degradation products have no folate activity.[1,22,23,29] It could be expected that irradiation will also yield inactive degradation products. No attempt was made yet to identify the degradation products, and one can consider that a decrease of FA content leads to a decrease of the folate activity of the food. In summary, an important irradiation effect on FA degradation was observed for all of the dose range studied (0.25−10 kGy), no matter the conditions assayed (atmosphere or pH) (Figure 2).

Table 1. D_{50} Values (kGy) of FA Aqueous Solutions at pH 6.8, 8.0, and 12.0 with O_2 and N_2

	pH		
	6.8	8.0	12.0
O_2	0.80	0.53	0.37
N_2	0.42	0.75	0.50

Irradiation Effect on FA Suspension, FA in Powder, and Wheat Flour Fortified with FA. The effect of E-beam treatment in a FA suspension was studied. Because FA is practically insoluble in water (0.01 mg/mL), the work solution of 0.1 mg/mL of FA resulted in dispersion instead of a solution. FA is only soluble and stable in dilute alkaline solution and dissolves but is unstable in acid medium.[9,29] Similar to FA in powder, FA in suspension (0.1 mg/mL in water) showed no significant difference between non-irradiated and irradiated samples (Figure 3). The high stability of FA in suspension to radiation processing remained similar from 0 to 5 kGy even if, in this case, an

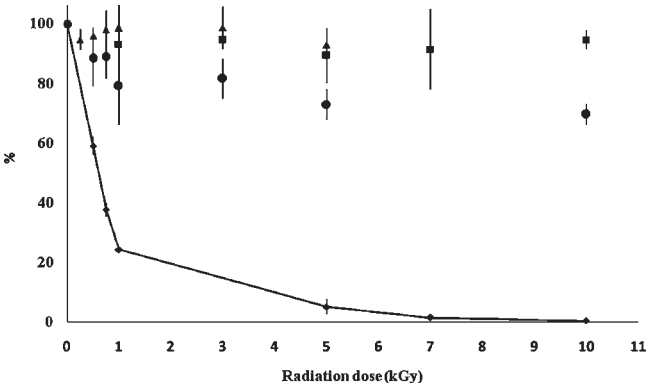

Figure 3. Recovery percentage of FA in powder, suspension, and solution: (◆) FA in solution, (■) FA in powder, (▲) FA in suspension, and (●) wheat flour fortified with FA.

important indirect effect because of water radiolysis could have happened, especially with higher doses.

To evaluate the moisture effect in dry samples, such as wheat flour and FA in powder, Karl Fischer titration was performed. In wheat flour samples, the water content averages 13% (m/m). Such a value is highly consistent with previously published data.[30] Even commercially available FA (in powder) contains on average 8.0−8.5% of water of hydration.[31] From our results, FA irradiated in powder, even containing 8% of water, did not present any statistically significant degradation over a dose range of 1−10 kGy (Figure 3). It suggests that, when non-solubilized, FA is not sensitive to irradiation treatment, at least up to 10 kGy. On the other hand, in the fortified wheat flour samples, the FA content showed a very slight degradation (less than 30%) when irradiated at doses of 5 and 10 kGy ($p < 0.05$). The higher moisture content and more certainly the food matrix, during irradiation and/or extraction, could have enhanced the irradiation effect in these samples. With regard to the loss of these synthetic vitamer at doses higher than 5 kGy, it should be noted that irradiation of grains, cereals, and flours aims for disinfestation. Required doses for this purpose remain below 1 kGy,[14,15] very low absorbed doses, which give no significant reduction of the content of the supplemented vitamer. As signalized by Müller et al.,[19] the relatively high radiation stability of folates contrasts with the observation that conventional processing and culinary preparation destroyed about 50% of the folate concentration in samples of total diet. Galán et al.,[20] studying the E-beam effect on hamburgers enriched with FA, found 20−30% FA reduction following irradiation with 2 kGy. Our study showed a similar decrease, nevertheless with a dose range much higher (5 and 10 kGy). This can be easily explained just by the high moisture content in hamburgers. Müller et al.[19] observed that the radiation stability of folates was higher in dehydrated spinach, cabbage, and Brussels sprouts than in the fresh material. It is worth saying that in-house fortified wheat flour showed similar quantitative results as those from a commercially available Brazilian wheat flour, showing that the FA radiodegradation does not depend upon the origin of the flour (data not shown).

It became evident that the destruction suffered by dry materials, even when in suspension, as a result of irradiation is much smaller than that in aqueous solution, where indirect effects predominate. Wheat flour fortified with FA showed no loss in its FA content with doses required for disinfestation (up to 1 kGy).

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