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# LC/MS/MS identification of some folic acid degradation products after E-beam irradiation

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ABSTRACT

Folates belong to the B vitamin group based on the parental compound folic acid (FA). They are involved in important biochemical processes like DNA synthesis and repair. FA is composed of a pteridine ring, p-aminobenzoic acid and glutamate moieties. The human metabolism is not able to synthesize folates and therefore obtain them from diet. FA, a synthetic vitamin, is used as a food fortificant because of its low price, relative stability and increased bioavailability compared to natural folate forms. FA is known to be a sensitive compound easily degradable in aqueous solution by ultraviolet and visible light towards various by-products. Irradiation is a process for preservation of foods that uses accelerated electrons, gamma rays or X-rays. Irradiation is proposed for the treatment of various food products, eliminating or reducing pathogens and insects, increasing the storage time and replacing chemical fumigants. This study concerns the identification of degradation products of FA after E-beam irradiation. FA aqueous solutions were irradiated with a Van de Graaff electrons beam accelerator (2 MeV, 100 μA current, 20 cm scan width, dose rate about 2 kGy/s). Applied doses were between 0 (control) and 10.0 kGy. Absorbed doses were monitored with FWT 60.00 radiochromic dosimeters.



## 1. Introduction

Folic acid (FA) is the parent compound of the folates water-soluble vitamins. As shown in Fig. 1, FA ($C_{19}H_{19}N_7O_6$, MW=441.4) is composed of a pteridine ring, p-aminobenzoic acid (PABA) and glutamate moieties. FA is the synthetic oxidized and most stable form of folates. FA is an orange–yellow, microcrystalline, almost odorless, tasteless powder (Lucock, 2000; Ball, 2006).

Humans do not synthesize folates and therefore have to get them from food. Folates are attracting considerable interest in the field of nutrition due to the growing amount of evidence pointing to the contribution of inadequate folate intake to severe health disorders such as neural tube defects (Tamura and Picciano, 2006), cardiovascular disease (Moat et al., 2004), neuropsychiatric disorders (Selhub et al., 2000) and some types of cancer (Kim, 2007).

The stability of folates during food processing is influenced by temperature, duration of heating, pressure, pH, radiation dose, oxygen, light, metal ions and antioxidants moieties (McKillop et al., 2002; Indrawati et al., 2004; Verlinde et al., 2008; Araujo et al., 2011). Degradation of this molecule may result from scission reaction such as cleavage of the $C^9$–$N^{10}$ covalent bond, which results in irreversible loss of vitamin activity (Murphy et al., 1976; McKillop et al., 2002).

Radiation treatment is a process exposing food to ionizing radiations such as gamma rays emitted from the radioisotopes $^{60}Co$, or high energy electrons and X-rays produced by machine sources. Depending on 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 (Farkas, 2006). Research showed that macronutrients such as proteins and carbohydrates are relatively stable at doses up to 10 kGy, and micronutrients, especially vitamins, may be sensitive to any food treatment (Villavicencio et al., 2000).

In this study, the stability of the synthetic form FA was investigated after E-beam irradiation in aqueous solution. The main purpose of this research was to perform a detailed study on FA degradation due to irradiation treatment. At first, degradation of FA after irradiation treatment was evaluated in a wide range of radiation doses to identify the radiation dose range of interest. Later on, predominant degradation products were analyzed and identified to investigate possible degradation mechanisms.

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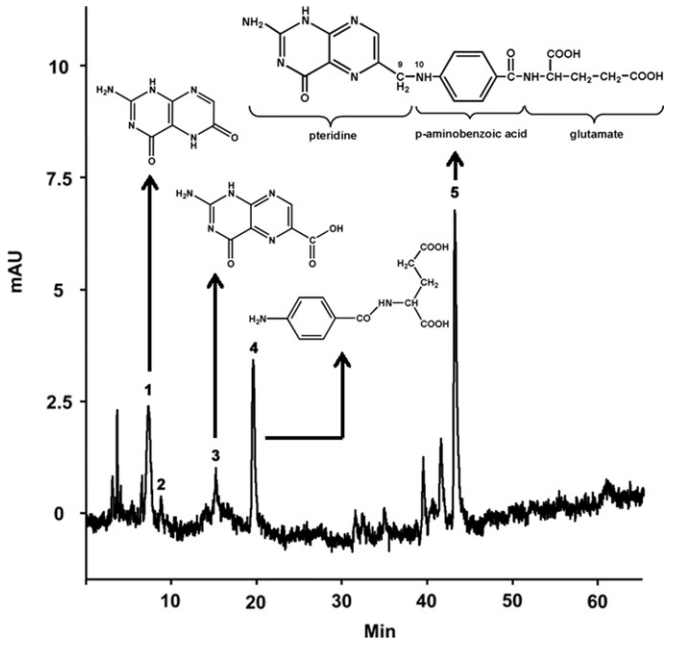


**Fig. 1.** LC/UV chromatogram of FA solution irradiated at 5 kGy and degradation products (1, 2, 3, 4) of folic acid (5).

## 2. Experimental

### 2.1. Materials

Methanol (MeOH) (Carlo Erba, Val de Reuil, France) and acetonitrile (ACN) (Sigma-Aldrich, Steinheim, Germany) were high-performance liquid chromatography (HPLC) grade. All other chemicals were of analytical grade. Acetic acid was purchased from Riedel-de-Haen/Fluka (Seelze, Germany). Sodium hydroxide (NaOH) was purchased from SDS (Peypin, France). FA and degradation products pterine-6-carboxilic acid (PCA), p-aminobenzoyl-L-glutamic acid (pABGA) and p-aminobenzoic acid (PABA) were obtained from Sigma-Aldrich (Steinheim, Germany). Xanthopterin monohydrate (XA) was purchased from Acros Organics (Geel, Belgium). Water was purified using a Synergy Milli-Q System (Millipore, Molsheim, France).

### 2.2. Samples

All folic acid standard solutions were prepared under subdued light, to protect folates from oxidative degradation induced by light. Solutions were prepared as following: a total of 200 mg of FA was added to 200 mL of water and solubilized by the addition of 10 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 11. All samples (4 mm thick) were packed in polyamide/polyethylene plastic sachets, sealed and labeled with their respective radiation doses. PABA and pABGA were dissolved and diluted in water pH 7.0 while PCA and XA were dissolved and diluted in alkaline water, all of them at a final concentration of 0.1 mg/mL.

### 2.3. Irradiation treatment

FA aqueous solutions 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), 0.24, 0.46, 0.72, 0.95, 2.9, 5.0, 7.0 and 10.1 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) (Kuntz et al., 1996). Dose uniformity of about 10% within the sample was achieved by the use of a 100 μm thick copper scattering foil (Kuntz et al., 1990). During irradiation, standard conditions for temperature and pressure were used (25 °C and 14.69 psi).

### 2.4. LC/MS/MS

Experimental work was performed using a Varian ProStar HPLC system (Palo Alto, CA) [210 binary HPLC pump, 410 AutoSampler and 335 diode array detection (DAD) UV–vis detector]. A Varian 1200L triple quadrupole mass spectrometer system (MS) (Varian, Les Ulis, France) was used for the identification of degradation products. High-purity nitrogen (Domnik Hunter, Villefranche sur Saône, France) was used as nebulizing gas, set at 46 psi, and as a drying gas, set at 200 °C. The spectrometer was operated in scan mode (70–500$m/z$) using positive electrospray ionization (ESI). Chromatographic separation was carried out on a Agilent-XDB Phenyl analytical column (250 mm × 4.6 mm; 5 μm particle size), whereas the eluate was split at the ratio of 1:5 before entering the MS. Mobile phase consisted of aqueous acetic acid (0.5%) and MeOH, at a flow rate of 1.0 mL/min. Gradient elution started at 100% aqueous acetic acid maintained for 10 min, followed by raising the MeOH concentration linearly to 60% within 56 min. MeOH concentration was maintained for 4 min. Subsequently, the MeOH concentration decreased to 0% in 1 min, and then the column was equilibrated at this condition for 9 min. An injection volume of 20 μL was used.

Confirmation analysis was performed using a high resolution mass spectrometer (HRMS) (Agilent Technologies, Massy, France) [G1312B binary pump, G1367C AutoSampler, DAD SL G1315C detector, MS 6520 Accurate Mass Q-TOF]. Chromatographic separation was carried out on a Thermo Hypersil Gold C8 analytical column (100 mm × 1.0 mm; 1.9 μm particle size). Mobile phase consisted of aqueous formic acid (0.05%) and ACN, at a flow rate of 0.1 mL/min. Gradient elution started at 100% aqueous formic acid maintained for 2 min, followed by raising the ACN concentration linearly to 95% within 6 min. ACN concentration was maintained for 6.5 min. Subsequently, the ACN concentration decreased to 0% in 0.5 min. An injection volume of 0.4 μL was used.

## 3. Results

Increasing absorbed radiation doses promoted a decrease in FA concentration, as noted by other authors (Müller and Diehl, 1996; Araujo et al., 2011). FA aqueous solutions also showed different chromatographic profiles depending on radiation doses applied. Low radiation doses (up to 1 kGy) caused mainly the appearance of less polar degradation products which present close $m/z$ to FA and surround FA retention time (Fig. 1, peak 5) (HPLC chromatograms not shown). On the other hand, radiation doses over 5 kGy promoted an intense FA breakdown, so smaller degradation fragments (low $m/z$) compared to FA $m/z$ of polar nature appeared (Fig. 1).

Three of these more polar degradation products due to E-beam treatment were clearly identified and meets to already known FA degradation products. Even at doses around 1 kGy these radiation degradation products could be detected. The first one, represented by the peak at retention time ($t_r$) 15.2 min yielded a $[M+H]^+$ of $m/z$ 208.0. This peak represented a $C^9$–$N^{10}$ bond cleavage, resulting in the formation of pterine-6-carboxilic acid (PCA) ($C_7H_5N_5O_3$) (Fig. 1, peak 3). It was also observed a $m/z$ 229.9, due to sodium adduct $[M+Na]^+$. The second one, at $t_r$=19.5 min, a $[M+H]^+$ of $m/z$ 267.0 was found representing the loss of the pteridine group

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**Table 1**
FA degradation products and their corresponding $t_r$, $m/z$ (MS), $m/z$ (HRMS) and empirical formula.

| Compound | Retention time (min) | MS $[M+H]^+$ | HRMS $[M+H]^+$ | Empirical formula |
|---|---|---|---|---|
| XA | 7.5 | 180.0 | 180.0518 | $C_6H_5N_5O_2$ |
| Unkown compound | 9.5 | 194.0 | 194.0676 | $C_7H_7N_5O_2$ |
| PCA | 15.2 | 208.0 | 208.0464 | $C_7H_5N_5O_3$ |
| PABA | 19.0 | 138.0 | 138.0549 | $C_7H_7NO_2$ |
| pABGA | 19.5 | 267.0 | 267.0992 | $C_{12}H_{14}N_2O_5$ |

moiety, arising the p-aminobenzoyl-L-glutamic acid (pABGA) ($C_{12}H_{14}N_2O_5$) (Fig. 1, peak 4). It was also observed $m/z$ 289.0, due to sodium adduct $[M+Na]^+$. The presence of sodium adducts is well known in mass spectrometry due to the current use of glass containers for solvents. In our case the presence of sodium adducts might be also due to the use of a NaOH solution to dissolve FA in water. Moreover, in the same mass spectrum, an $m/z$ of 119.9 was observed representing the loss of the glutamate moiety. Also trace of $m/z$ 138.0 $[M+H]^+$ was found in irradiated FA aqueous solutions ($t_r$=19.0 min). It corresponds to the loss of both pteridine ring and glutamate moieties, resulting in p-aminobenzoic acid (PABA) ($C_7H_7NO_2$). The identification of PCA, PABA and pABGA in irradiated FA solutions were confirmed by comparing their $t_r$, UV and MS/MS spectra with these degradation products standards in LC/UV/$MS^2$ chromatograms (data not shown).These evidences were also confirmed by high resolution mass spectrometry, resulting in a $m/z$ of 208.0464, 138.0549 and 267.0992 $[M+H]^+$, respectively to PCA, PABA and pABGA (Table 1).

Besides the known degradation products, other peaks were also observed in LC/UV/MS chromatograms (Fig. 1). One of them ($t_r$=7.5 min), presented a $m/z$ of 180.0 $[M+H]^+$ (Fig. 1, peak 1). High resolution mass spectrometry showed that this new compound has an empirical formula of $C_6H_5N_5O_2$ and $m/z$ of 180.0518, which was supposed to be xanthopterin. This hypothesis was confirmed by analysis of xanthopterin standard which showed the same $t_r$, UV and MS/MS spectra in LC/UV/$MS^2$ chromatograms. Another neo-formed product was detected at $t_r$=9.6 min and presented a $m/z$ 194.0 $[M+H]^+$, 175.9 $[M+H-H_2O]^+$ and $m/z$ 215.9 $[M+Na]^+$ (Fig. 1, peak 2). From HRMS analysis, it was found an empirical formula of $C_7H_7N_5O_2$ and a $m/z$ 194.0676 $[M+H]^+$ (Table 1).

## 4. Discussion

Some of our findings are already well-known FA breakdown products. Tannenbaum et al. (1985) have already showed that alkaline hydrolysis under aerobic conditions promotes oxidative cleavage of the FA molecule to yield pABGA and PCA. Vora et al. (2002), in a study of the thermal stability of FA, proposed that first folic acid loss the glutamate moiety and then pterin and PABA decomposes in an overlapping mechanism.

Our results are in accordance with those found after photolysis of FA. The first study published by Lowry et al. (1949), had already proposed that after photolysis FA finally degrades to pABGA and PCA. Saxby et al. (1983) evaluated the effect of sulfur dioxide ($SO_2$) on the stability of FA in aqueous solution and in beer, both at pH 5. They stated that FA stored in the light degraded to a mixture of three compounds, PCA, pABGA and probably 6-methylpterin and $SO_2$ played a protective role. In beer, the presence of $SO_2$ had no effect on the degradation of FA, which decomposed finally to the same products. No approach was done with regard to the type of light applied in these previous studies. Afterwards, several studies were performed (Akhtar et al., 1997, 1999, 2003; Thomas et al., 2000; Off et al., 2005; Vorobey et al., 2006; Dántola et al., 2010) focusing on the photolysis of FA under UV light. They have proposed that after UV irradiation, FA first undergoes a cleavage at the $C^9$–$N^{10}$ bond with formation of pABGA and 6-formylpterin (FPT) ($C_7H_5N_5O_2$, MW: 191). Further UV irradiation leaded to photo-oxidation of the 6-formyl group of FPT and thus formation of PCA. Also, both photoproducts, FPT and PCA, can act as sensitizers in the photodegradation of FA. In this study, after E-beam treatment no trace of FPT was detected, probably due to the high energy involved in E-beam treatment that accelerated PCA formation. E-beam treatment promoted FA degradation into similar degradation products as UV treatment, but also new breakdown products. That is understandable because E-beam processing is an in-depth treatment, more energetic and with higher dose rates than UV treatment.

A few works have already tried to elucidate the degradative process of FA after irradiation treatment, yielding mainly PCA and pABGA as degradation products, similarly to our data. Nakken and Pihl (1966) studied the X-ray induced degradation of folic acid, p-aminobenzoic acid conjugates, N-(p-aminobenzoyl)glutamic acid and N-(p-aminobenzoyl)glycine under pH 7.4. They also proposed that one susceptible point of attack for radiation-induced radicals might be the $C^9$–$N^{10}$ linkage, resulting in pABGA and PCA products. Also 3-OH-pABGA and o-amino-phenols were found. In another study, Kesavan et al. (2003) evaluated the effect of total body gamma irradiation (TBI) (2, 5, 7 Gy) in mice. They also observed that after TBI the folate molecule was split into pterin and pABGA due to oxidative damage.

Further investigations are under analysis in order to enlighten the chemical structure of other FA radiation degradation products as well as the mechanism of FA degradation after E-beam treatment.

## 5. Conclusions

E-beam treatment with radiation doses over 1 kGy promoted an intense FA breakdown. FA molecule was cleaved not only into already known degradation products PCA, pABGA and PABA, but also into other products. One of them was identified as xanthopterin, a new radio-induced breakdown product.

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