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Mechanisms of folate losses during processing: Diffusion vs. heat degradation

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A B S T R A C T

Though folates are sensitive to heat treatments, leaching appears to be a major mechanism involved in folate losses in vegetables during processing. The aim of our study was to study folate diffusivity and degradation from spinach and green beans, in order to determine the proportion of each mechanism involved in folate losses.

Folate diffusivity constant, calculated according to Fick's second law (Crank, 1975), was $7.4 \times 10^{-12} \text{ m}^2/\text{s}$ for spinach and $5.8 \times 10^{-10} \text{ m}^2/\text{s}$ for green beans, which is the same order of magnitude as for sugars and acids for each vegetable considered. Folate thermal degradation kinetics was not monotonous in spinach and green beans especially at 45 °C and did not follow a first order reaction. The proportion of vitamins changed markedly after thermal treatment, with a better retention of formyl derivatives. For spinach, folate losses were mainly due to diffusion while for green beans thermal degradation seemed to be preponderant.

Keywords:

Vitamins
Folate
Vegetables
Process
Leaching
Heat degradation
Kinetics
Fick's second law

1. Introduction

Folate is the generic term used for different water-soluble vitamins which differ by the nature of carbon groups linked to nitrogen 5 or 10, the oxidative state and the length of the glutamate tail. They are involved in the "one carbon metabolism" especially being donor of methyl group during DNA synthesis. It is well established that folates can protect against neural tube defects (Czeizel & Dudás, 1992) and neurodegenerative diseases (Snowdon, Tully, Smith, Riley, & Markesbery, 2000). Folates are also involved in the methylation of homocysteine, which is one risk factor for heart diseases (Robinson, 2000).

One of the main contributors for folate intake are vegetables, and particularly green vegetables, which represent circa 40% of the folate intake in the French diet (Lafay, 2009). In France, authorities recommend an intake of folate from 300 µg per day for women to 330 µg per day for men, with an increase to 1 mg per

day during pregnancy (ANSES, 2012). However, there is a gap between the real and the recommended intake from around 20% for women to 15% for men (Lafay, 2009).

Evolution of lifestyles means that most fruits and vegetables are consumed after processing, whether domestic processing (cooking, heating, microwaves) or industrial processing, such as canning or freezing, hence there is a need to better understand the impact of processing on folate content.

Folate losses from spinach during boiling or blanching represent 20–80% of initial folates and from 0% to 20% in green beans (Delchier, Reich, & Renard, 2012; Desouza & Eitenmiller, 1986; Klein, Lee, Reynolds, & Wangles, 1979; McKillop et al., 2002; Melse-Boonstra et al., 2002). However, steaming and microwave cooking did not cause folate losses (Delchier et al., 2012; Klein et al., 1979; McKillop et al., 2002). Few studies measured folates in cooking liquids but Delchier et al. (2012) showed that leached folates represent half of folate losses from fresh spinach and the whole of folates losses from frozen spinach and green beans, after boiling in water. Data concerning the impact of industrial processing on folate losses is really scarce. Our previous study showed that blanching had no effect on folate loss both during the spinach freezing process and the green beans canning process. Losses occur

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during the washing step for spinach and after sterilization for green beans, with folate found in the covering liquid (Delchier et al., 2013).

This study on folate loss during industrial processing led us to suspect that diffusion may play a major role in folate loss, especially when heating steps are limited.

Therefore, this study aims to determine the relative importance of diffusion and thermal degradation of spinach and green beans during heat treatments. For this, two parallel experiments were set up: one in which vegetables were only subjected to heat, and one in which they were subjected to heat and diffusion.

2. Materials and methods

2.1. Plant material

2.1.1. Diffusion

Fresh spinach and green beans were bought at a local supermarket on the day of the experiments, or stored at 4 °C for maximum of 48 h after purchase. Spinach and green beans were first blanched in phosphate buffer pH 7 (0.01 mol/l) or in citrate phosphate buffer pH 5 (0.01 mol/l) for 10 min at 100 °C with solid-liquid ratio of 50 and 100 g/l, respectively, in order to inactivate enzymes and destroy cell compartmentalization. After blanching, spinach and green beans were drained, weighted and immediately put into a large receptacle (with the same solid-liquid ratio of 50 g/l), to start the diffusion.

2.1.2. Thermal degradation

Purees were prepared from spinach and green beans stored in cans bought at local supermarket in two batches for each temperature condition. Cans were opened and vegetables were drained. 200 g of vegetables were put into 400 ml of water and ground with an UltraTurax (S25 18G, IKA, Staufen, Germany) at 13,000 rpm for 1 min. Spinach purees were diluted, to facilitate stirring during the time course. For this purpose, 50 ml of water was added to 50 ml of spinach puree.

2.2. Time course experiments

2.2.1. Diffusion

Diffusions experiments were carried out in phosphate buffer pH 7 (0.01 mol/l) or citrate phosphate buffer pH 5 (0.01 mol/l), under stirring. Temperature and pH were monitored and controlled all along the time course, which were performed for three temperatures (25, 45 and 65 °C) and at pH 5 and pH 7 for 4 h. At pH 7, three batches of spinach and green beans were independently studied and two batches at pH 5.

For each kinetic point, an aliquot of 35 g of spinach or green beans was collected and directly stabilized by freezing in liquid nitrogen, and stored at -80 °C until analysis. The folate content was determined in the vegetables at each point along the time course.

2.2.2. Thermal degradation

Heat degradation was carried out in a beaker immersed in a water bath. Purees were stirred all along the experiments by a propeller stirrer of 55 mm diameter turning at 600 rpm (VOS 16, VWR, Fontenay sous bois, France). Time courses were performed in two independent batches for three temperatures: 45, 65 and 85 °C. Purees were heated and kinetics started when they were at the desired temperature. 10 ml of puree were sampled at different points for 4 h and directly put at -80 °C.

2.3. Modelling

2.3.1. Diffusion

Diffusivity constant (D) was calculated for folates, sugars and acids according to Fick's second law Eq. (1):

$$\frac{\partial C}{\partial t} = -D \frac{\partial^2 C}{\partial r^2} \quad (1)$$

where C represents the concentration, t the time and r a characteristic distance.

Spinach leaves were considered as a plane sheet where Fick's second law solution, given by Crank (1975), is Eq. (2):

$$\frac{C(t) - C_\infty}{C_0 - C_\infty} = 1 - \sum_{n=0}^{\infty} \frac{8}{(2n+1)^2 \pi^2} \exp\left(-\frac{D(2n+1)^2 \pi^2 t}{4l^2}\right) \quad (2)$$

where $C(t)$ is the concentration at time t , C_0 is the initial concentration and C_∞ the concentration at infinite time, D the diffusivity constant and l the half thickness of the plane.

In the case of green beans, the diffusivity constant was determined according to the cylinder solution given by Crank (1975) Eq. (3):

$$\frac{C(t) - C_\infty}{C_0 - C_\infty} = 1 - \sum_{n=1}^{\infty} \frac{4}{a^2 \alpha_n^2} \exp(-D\alpha_n^2 t) \quad (3)$$

where $C(t)$ is the folates concentration at t time, C_0 is the initial concentration and C_∞ the concentration at infinite time, D the diffusivity constant and a the radius. In this equation α_n is the root of Bessel function of order 0 Eq. (4).

$$J_0(a\alpha_n) = 0 \quad (4)$$

The model was adjusted by maximizing the correlation coefficient, r^2 , calculated as follow Eq. (5):

$$r^2 = 1 - \frac{\left(\sum (Exp - Th)^2\right)}{\left(\sum (Exp - m_{Th})^2\right)} \quad (5)$$

where Exp is the experimental concentration; Th is the theoretical data obtained by modelling, and m_{Th} is the mean of theoretical data obtained by modelling.

2.3.2. Thermal degradation

Linearization of folate thermal degradation was carried out according to the mean of the two batches for each temperature studied, using a first order with partial conversion model, as described below Eq. (6):

$$\ln\left(\frac{C}{C_0}\right) = -kt \quad (6)$$

where C is the folate concentration, C_0 is the initial folate concentration, k is the degradation rate constant and t the time.

2.4. Analytical procedures

2.4.1. Folate measurement

2.4.1.1. Total folates content. Total folate content was determined by HPLC with fluorimetric detection. After extraction all folate vitamers were deconjugated into mono and diglutamate, reduced and methylated into 5-CH₃-H₄folates. The latter were purified from the extract by affinity chromatography using Folate Binding Protein, and quantified by RP-HPLC with fluorimetric detection (RF-1AXL, Shimadzu Inc., Kyoto, Japan). For experimental details, see Delchier et al. (2013).

2.4.1.2. Stable isotope dilution assay. Before extraction, labelled standards of folate vitamers were added and all folates were

deconjugated into their monoglutamate forms. Vitamers were purified on SPE SAX cartridges after adding acetonitrile (10 ml) and centrifugation. Folate analysis was carried out on an HPLC (Shimadzu Inc., Kyoto, Japan) coupled with a triple quadrupole mass spectrometer (API 4000 Q-Trap, AB-Sciex, Foster City, CA, USA). A Pro-C18 HPLC-column (150 × 3, 3 μm, 130 Å, YMC, Japan) with water plus 0.1% (v/v) formic acid (A) and acetonitrile plus 0.1% (v/v) formic acid (B) as mobile phases was used. The gradient started at 5% B. After a linear increase to 10% B in 5 min and holding this condition for 5 min, another linear increase to 15% B during 10 min and to 50% B in 2 min, which was maintained for 2 min. Within 2 min B was decreased linearly to 5% and the column was equilibrated for 9 min.

Concentration of the single vitamers in the food samples was calculated using the response factors reported recently (Ringling & Rychlik, 2013). For more experimental details see Ringling and Rychlik (2013).

2.4.2. Acid and sugar measurement

Acid and sugar concentrations were determined by spectrophotometry (Xenius, Safas, Monaco) by determining concentrations of NADPH and NADH respectively, at 340 nm using enzymatic kits. Enzymatic kits for citric acid (reference: 10.139.076), malic acid (reference: 10.139.068), glucose (reference: 10.716.251), fructose (reference: 10.139.106) and sucrose (reference: 10.716.251) were obtained from R-Biopharm (Darmstadt, Germany).

Sugars and acids were extracted from 200 mg of spinach or green bean powder, to which 1 ml of deionised water was added. The mix was stirred for 1 min and then centrifuged (Bioblock Scientific 1K15, Illkirch, France) for 10 min at 7400g at 4 °C. 5 μl of supernatant was pipetted into the microplate, and 250 μl of enzymatic reagent were added.

The concentrations were determined against external calibration from 0 to 1 g/l for citric and malic acids, from 0 to 2 g/l for fructose and glucose and from 0 to 4 g/l for sucrose.

3. Results

3.1. Thermal degradation

In spinach, for each temperature, the folate concentration decreased over time, with the appearance of a plateau at 120 min at 45 °C, 60 min at 65 °C and 30 min at 85 °C (Fig. 1). However, this decrease was not monotonous, and concentration increased particularly between 10 and 20 min at 45 °C. For each temperature, the difference in concentrations between the two batches was relatively low. For all temperatures, degradation of the folates under study was not complete, and a plateau was reached with a C_{∞}/C_0 ratio of 40% at 45 °C, 42% at 65 °C and 48% at 85 °C.

In green beans, evolution of folate concentrations at 45 °C showed an increase in the first 20 min, which was more marked for batch 2, followed by a decrease until reaching a plateau at 120 min (Fig. 1). At higher temperatures, the concentration decrease was monotonous, except at 60 min, and 65 °C, for the two independent kinetics. This decrease reached a plateau at 90 min for the two batches at 65 °C and at 20 min for the two batches at 85 °C. Variability between batches for each temperature was low. Overall, temperature accelerated folate degradation, the plateau being reached more rapidly at 85 °C than at 65 °C or 45 °C. However, folate degradation was not complete; the level of the C_{∞}/C_0 ratio was 20% both at 45 °C and 65 °C.

Folate thermal degradation kinetics were modeled using first order with partial conversion. However, this model was not satisfying at 45 °C, where the folate concentration increased in the first minutes of the kinetics.

3.2. Folate losses during diffusion

3.2.1. Total folate

Total folate concentration was determined in vegetables by HPLC with fluorimetric detection after precolumn derivatization, and expressed as 5-CH₃-H₄folate in mg/kg of fresh weight.

For spinach, the folate concentration at the beginning of the experiments, for both pH and the three temperatures studied, varied slightly between different batches. In all conditions, the folate concentration first decreased rapidly over time. This decrease was exponential until reaching a plateau after 60 min (data not shown). In green beans, the folate concentration at the beginning of the diffusion time course was the same (about 0.2 mg/kg expressed in fresh material) for both pH and temperatures studied. The decrease in folate concentration was much more dependent on temperature. Thus, at pH 5 and 25 °C the folate content was almost stable. However, it decreased at 45 °C and more markedly at 65 °C. Folate decrease appeared to be exponential until reaching a plateau at 120 min, at least at 65 °C (data not shown).

The decrease in folate was faster for spinach than for green beans at the beginning of the time course. The plateau was also reached faster for spinach than for green beans with a higher level for green beans than for spinach. Indeed, the residual ratio in spinach (Table 1), expressed by the C_{∞}/C_0 ratio at the plateau, was (on average) 26% of the initial concentration at pH 7 and in the same order of magnitude at pH 5. At pH 7, temperature had no effect on residual ratio while at pH 5 temperature had an effect especially at 65 °C. For green beans, the residual ratios (C_{∞}/C_0) were on average 62% of the initial concentration at pH 7 and 72% of the initial concentration at pH 5 (Table 1). Temperature seemed to have an effect on the residual ratio both at pH 7 (from 0.7 at 25 °C to 0.41 at 65 °C) and at pH 5 (from 1.17 at 25 °C to 0.43 at 65 °C).

Finally, the folate residual ratios (C_{∞}/C_0) were lower than those observed during the thermal degradation studies (40% at 45 °C and 42% at 65 °C), for spinach at pH 5. For green beans, these residual ratios were higher than those observed in thermal degradation at pH 5 (20% at 45 °C and 65 °C), the pH corresponding to that of spinach and green beans purees used during thermal degradation kinetics.

3.2.2. Folate derivatives

Concentrations of folate derivatives (in mg/kg as fresh weight), in the initial sample and in the final sample of the diffusion kinetic studies were determined by stable isotope dilution assays. Results are presented in Table 2 for spinach and Table 3 for green beans.

Both in spinach and green beans, the main compound was 5-CH₃-H₄folate which represented about 70% of folates at the beginning of diffusion and also revealed the highest loss of all derivatives during diffusion. At pH 7, 5-CH₃-H₄folate residual ratios (C_{∞}/C_0) in spinach decreased with increasing temperature. The same trend was observed with higher residual ratio (C_{∞}/C_0) at pH 5. For green beans, 5-CH₃-H₄folate residual ratio (C_{∞}/C_0) decreased with temperature increase at pH 7 and pH 5.

The second main class of folates derivatives was formyl derivatives. 10-HCO-PteGlu and 5-HCO-H₄folate represented 6% to 16% in spinach and 7% to 12% in green beans respectively. 5-HCO-H₄folate residual ratios (C_{∞}/C_0) increased with the temperature increase both in spinach and green beans between pH 7 and pH 5. 10-HCO-PteGlu residual ratios (C_{∞}/C_0) were quite stable at 25 °C both at pH 7 and 5 while it increased with rising temperature both at pH 7 and 5.

Residual ratios (C_{∞}/C_0) of minor derivatives (PteGlu, H₄folate, 5,10-CH⁺-H₄folate and 10-HCO-H₂folate) were very low and appeared quite variable during kinetics, both for spinach and green beans.

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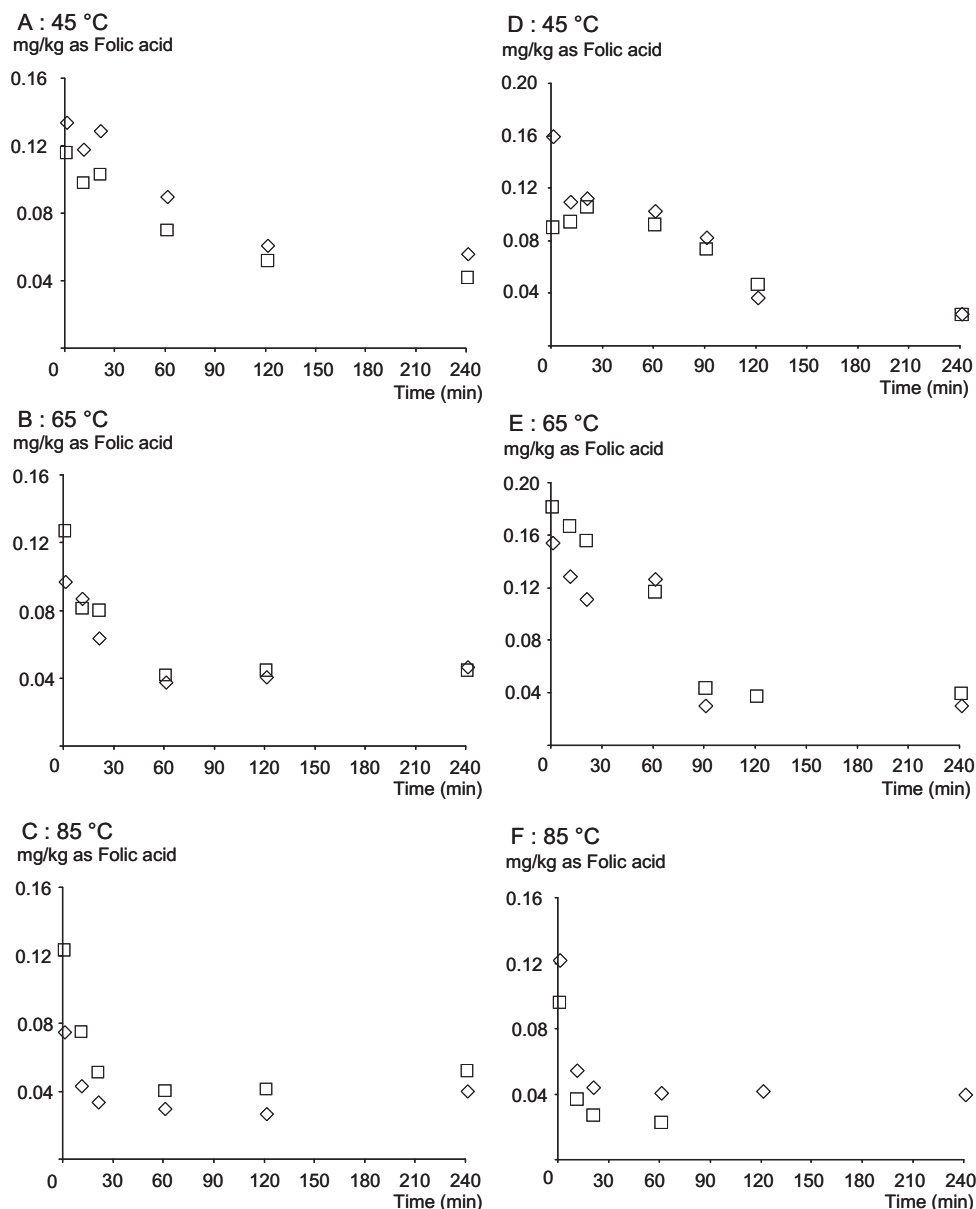


Fig. 1. Folates' thermal degradation in spinach and green beans purees. The left part of the graph represents the thermal degradation of total folates expressed as folic acid in mg/kg of fresh weight at 45 °C (A), 65 °C (B) and 85 °C (C) in spinach under atmospheric oxygen conditions. The right part of the graph represents the thermal degradation of total folates (mg/kg of fresh weight) at 45 °C (D), 65 °C (E) and 85 °C (F) under atmospheric oxygen conditions in green beans. Empty lozenges correspond to data from batch 1 and empty squares represent data from batch 2.

Finally, C_{∞}/C_0 obtained by determining concentrations by HPLC with fluorimetric detection were globally higher than those obtained by the sum of concentrations of derivatives (as folic acid) by the stable dilution assay, especially at 65 °C and pH 7. Analytical imprecision could be involved in this variation, especially by an over expression of concentrations resulting from the sample preparation for HPLC with fluorimetric detection.

3.3. Diffusion modelling

Modelling of experimental data was carried out according to Fick's second law for two reasons:

- Fick's second law is usually applied for describing of diffusion phenomena of water soluble compounds.

- The evolution of concentrations in the vegetables along the time course, with an exponential initial decrease followed by a plateau, corresponded to Fick's second law model both for plate (with an average thickness of spinach leaves of $2.74 \pm 0.8 \times 10^{-4}$ m) and cylinder (with an average green beans diameter of $6.66 \pm 1.36 \times 10^{-3}$ m).

The modeling was performed in the same way for all conditions of pH and temperature studied, both for folate, sugar and acid diffusion from spinach and green beans. An example is presented in Fig. 2, diffusion of folates for spinach at pH 7 and 45 °C (A) and for green beans at pH 7 and 65 °C (B), where lozenges represent experimental data and the line represents the diffusion model.

Model fitting was satisfactory both for spinach and green beans where the exponential shape curve was in accordance with

Table 1
 Diffusivity and C_{∞}/C_0 of folates, sugars and acids in spinach and green beans.

	pH	θ (°C)	Folates		Glucose		Fructose		Sucrose		Malic acid		Citric acid	
			D	C_{∞}/C_0	D	C_{∞}/C_0	D	C_{∞}/C_0	D	C_{∞}/C_0	D	C_{∞}/C_0	D	C_{∞}/C_0
Spinach	7	25	$7 \pm 2.1 \times 10^{-12}$	0.28 ± 0.07	3.0×10^{-12}	0.50	∅	∅	∅	∅	3.5×10^{-12}	0.49	∅	∅
		45	$7 \pm 1.7 \times 10^{-12}$	0.23 ± 0.03	1.0×10^{-11}	0.30	∅	∅	∅	∅	6.0×10^{-12}	0.19	∅	∅
		65	$6 \pm 1.1 \times 10^{-12}$	0.28 ± 0.12	∅	0.86	∅	∅	∅	∅	2.5×10^{-11}	0.52	∅	∅
	5	25	$6 \pm 2.1 \times 10^{-12}$	0.33 ± 0.01	6.0×10^{-12}	0.54	∅	∅	∅	∅	3.5×10^{-12}	0.57	∅	∅
		45	9×10^{-12} *	0.38 ± 0.14	8.0×10^{-12}	0.02	∅	∅	∅	∅	5.5×10^{-12}	0.55	∅	∅
		65	$8 \pm 1.4 \times 10^{-12}$	0.12 ± 0.04	9.5×10^{-12}	0.27	∅	∅	∅	∅	2.5×10^{-11}	0.27	∅	∅
Green Beans	7	25	$7 \pm 1.1 \times 10^{-10}$	0.73 ± 0.03	3.0×10^{-10}	0.61	1.5×10^{-10}	0.57	1.0×10^{-09}	0.51	1.0×10^{-09}	0.87	9.0×10^{-09}	0.05
		45	$8 \pm 1.7 \times 10^{-10}$	0.70 ± 0.06	3.0×10^{-10}	0.52	1.5×10^{-10}	0.55	2.5×10^{-10}	0.51	5.0×10^{-10}	0.44	9.0×10^{-09}	0.06
		65	$6 \pm 2.0 \times 10^{-10}$	0.41 ± 0.04	2.5×10^{-10}	0.39	6.0×10^{-10}	0.39	5.0×10^{-09}	0.63	2.5×10^{-10}	0.36	5.0×10^{-09}	0.03
	5	25	∅	1.17 ± 0.21	1.7×10^{-10}	0.71	3.0×10^{-10}	0.75	1.0×10^{-07}	0.61	5.0×10^{-10}	0.75	4.0×10^{-09}	0.07
		45	$5 \pm 1.4 \times 10^{-10}$	0.59 ± 0.08	1.5×10^{-10}	0.48	2.1×10^{-10}	0.43	5.0×10^{-10}	0.44	3.5×10^{-10}	0.51	5.0×10^{-09}	0.06
		65	3×10^{-10} *	0.43	1.5×10^{-10}	0.47	2.0×10^{-10}	0.38	5.0×10^{-10}	0.55	2.0×10^{-10}	0.48	5.0×10^{-09}	0.06

D corresponds to the diffusivity constant calculated for folates, glucose, fructose, sucrose, malic acid and citric acid in m^2/s . For the folate diffusivity constant, results are expressed as mean \pm standard deviation.

C_{∞}/C_0 corresponds to the residual concentration calculated for folates, glucose, fructose, sucrose, malic acid and citric acid. For folates, C_{∞}/C_0 is expressed as mean \pm standard deviation.

Diffusivity constants for folates were calculated from concentrations expressed as 5-CH₃-H₄folate monoglutamate (after conversion of all derivatives).

θ Corresponds to the temperature in degree Celsius.

∅ No data.

* No standard deviation was calculated for those samples.

Table 2
 Residual concentration (C_{∞}/C_0) of folate derivatives in spinach during diffusion kinetics.

pH	θ	Time	5-CH ₃ -H ₄ folate	5-HCO-H ₄ folate	10-HCO-PteGlu	H ₄ folate	PteGlu	5,10-CH ⁺ -H ₄ folate	10-HCO-H ₂ folate	Total folate (as folic acid)
7	25	t_0	0.302	0.057	0.022	0.015	0.003	0.003	0.010	0.395
		t_{180}	0.069	0.007	0.005	0.001	0.001	0.000	0.003	0.083
		C_{∞}/C_0	0.22	0.12	0.24	0.07	0.35	0.07	0.26	0.21
	45	t_0	0.189	0.010	0.027	0.004	0.001	0.000	0.010	0.230
		t_{240}	0.017	0.002	0.003	0.001	0.001	0.000	0.001	0.024
		C_{∞}/C_0	0.09	0.18	0.12	0.41	0.58	0.42	0.07	0.11
	65	t_0	0.137	0.027	0.020	0.005	0.002	0.001	0.010	0.193
		t_{240}	0.002	0.001	0.001	0.000	0.001	0.000	0.000	0.005
		C_{∞}/C_0	0.01	0.05	0.06	∅	0.31	∅	∅	0.03
5	25	t_0	0.280	0.064	0.013	0.012	0.002	0.002	0.003	0.361
		t_{240}	0.102	0.056	0.011	0.004	0.002	0.002	0.003	0.171
		C_{∞}/C_0	0.36	0.86	0.86	0.33	0.76	0.85	0.86	0.48
	45	t_0	0.344	0.109	0.008	0.039	0.002	0.003	0.002	0.484
		t_{240}	0.051	0.045	0.008	0.002	0.002	0.000	0.002	0.105
		C_{∞}/C_0	0.14	0.41	1.08	0.05	0.81	0.02	1.24	0.22
	65	t_0	0.350	0.126	0.009	0.035	0.003	0.003	0.003	0.506
		t_{240}	0.002	0.036	0.013	0.002	0.001	0.000	0.001	0.052
		C_{∞}/C_0	0.007	0.28	1.41	0.05	0.48	0.01	0.41	0.10

Results are expressed in mg/kg of fresh weight, in blanched spinach (t_0) and blanched spinach after diffusion (t_{180} or t_{240}).

C_{∞}/C_0 is the ratio between the initial concentration and the final concentration.

θ Corresponds to the temperature in degree Celsius.

Significance of italic values correspond to the ration C_{∞}/C_0 .

experimental data (Fig. 2). In order to validate the model, correlation coefficients were maximized, both for spinach and green beans. The correlation coefficients considering folate diffusion were between 0.87 and 0.99 for spinach and between 0.77 and 0.99 for green beans. For sugars and acids, the correlation coefficients varied from 0.82 to 0.99 for spinach and from 0.93 to 0.99 for green beans.

The high level of the correlation coefficients and the correlation between experimental data and model show an adequacy between the model calculated and the phenomena observed both for folates, sugars and acids in spinach and green beans.

3.3.1. Sugars and acids

Acids and sugars are water-soluble molecules and stable at pH 5 and 7 and temperatures from 25 to 65 °C, which were the conditions used in our study.

For spinach, only glucose and malic acid presented a sufficient initial concentration for relevant model fitting. Therefore, the diffusivity constant was calculated in spinach only for these two substances. The concentration of glucose and malic acid in spinach decreased until a plateau at about 60 min. For both glucose and malic acid, temperature and pH did not have an effect on residual ratios.

For both pH and temperature the concentration of sugars decreased in green beans until reaching a plateau at about 120 min. Plateau levels (C_{∞}/C_0) decreased with temperature at pH 7, and between 25 and 45 °C at pH 5, for glucose (Table 1). The residual ratio (C_{∞}/C_0) for fructose decreased with temperature both at pH 7 and 5, while the sucrose residual ratio appeared stable. There was an effect of temperature on the reduction of the residual ratio (C_{∞}/C_0) of malic acid. In addition, it appeared that residual ratios (C_{∞}/C_0) of malic acid were higher at pH 5 than at pH 7 and at 45 and at 65 °C.

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Table 3
 Folate derivatives concentration and C_{∞}/C_0 ratios in green beans during diffusion kinetics.

pH	θ	Time	5-CH ₃ -H ₄ folate	5-HCO-H ₄ folate	10-HCO-PteGlu	H ₄ folate	PteGlu	5,10-CH ⁺ -H ₄ folate	10-HCO-H ₂ folate	Total folates (as folic acid)
7	25	t_0	0.543	0.13	0.042	0.028	0.66	0.008	0.020	1.404
		t_{210}	0.353	0.07	0.038	0.005	0.001	0.002	0.014	0.465
		C_{∞}/C_0	0.65	0.54	0.91	0.18	0.21	0.28	0.69	0.33
	45	t_0	0.517	0.06	0.058	0.016	0.002	0.003	0.026	0.651
		t_{240}	0.216	0.04	0.043	0.009	0.002	0.003	0.015	0.315
		C_{∞}/C_0	0.41	0.73	0.73	0.55	0.81	1.02	0.56	0.48
	65	t_0	0.330	0.06	0.060	0.009	0.001	0.002	0.020	0.463
		t_{240}	0.027	0.04	0.039	0.007	0.002	0.006	0.012	0.128
		C_{∞}/C_0	0.08	0.67	0.65	0.80	2.35	2.36	0.59	0.28
5	25	t_0	0.342	0.07	0.032	0.014	0.001	0.002	0.009	0.449
		t_{180}	0.362	0.07	0.036	0.005	0.001	0.002	0.013	0.464
		C_{∞}/C_0	1.05	0.97	1.13	0.35	0.74	1.10	1.37	1.03
	45	t_0	0.541	0.07	0.039	0.012	0.001	0.003	0.021	0.661
		t_{240}	0.112	0.05	0.037	0.008	0.002	0.000	0.007	0.210
		C_{∞}/C_0	0.20	0.74	0.92	0.63	1.38	0.10	0.35	0.32
	65	t_0	0.448	0.09	0.040	0.014	0.001	0.003	0.013	0.582
		t_{240}	0.008	0.05	0.048	0.003	0.001	0.000	0.004	0.104
		C_{∞}/C_0	0.01	0.52	1.20	0.22	0.92	0.04	0.27	0.18

Results are expressed in mg/kg of fresh weight, in blanched green beans (t_0) and blanched green beans after diffusion (t_{180} or t_{240}).

C_{∞}/C_0 is the ratio between the initial concentration and the final concentration.

θ Corresponds to the temperature in degree Celsius.

Significance of italic values correspond to the ration C_{∞}/C_0 .

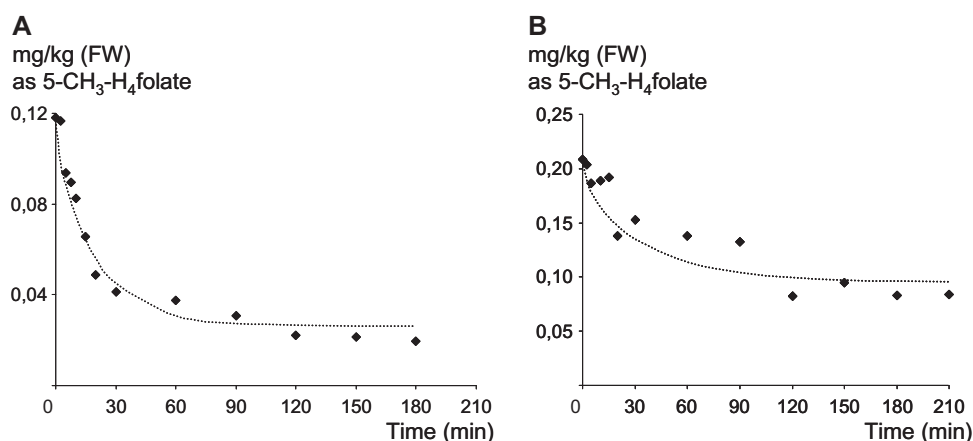


Fig. 2. Folate diffusion from spinach and green beans. On the left graph (A) experimental and modelling data for folate diffusion from spinach at pH 7 and 45 °C are presented and on the right graph (B) the respective data for folate diffusion from green beans at pH 7 and 65 °C. Experimental concentrations of folates during diffusion are expressed in mg/kg of fresh weight in equivalent 5-CH₃-H₄folate monoglutamate and represented by full lozenges. Concentrations determined by second Fick's law model are represented by the dotted line. Right graph (A).

For both spinach and green beans, diffusion of sugars and acids was determined as the mean of three batches for pH 7 and two batches for pH 5 for the three temperatures studied.

For spinach, the diffusivity constant determined was on average $5.5 \times 10^{-12} \text{ m}^2/\text{s}$ both for sugars and acids (Table 1). Glucose, fructose and sucrose from green beans showed a similar diffusivity constant, therefore only glucose, the most abundant compound, is presented. The temperature and pH did not seem to have any influence on the rate of diffusion of sugars in green beans, which was on average $6.1 \times 10^{-10} \text{ m}^2/\text{s}$.

Diffusion of acids (citric and malic acids) from green beans showed diffusivity constants in the same order for both acids at all temperatures and pH, on average about $4.6 \times 10^{-10} \text{ m}^2/\text{s}$ (Table 1). However, plateau levels were very different. Citric acid was almost completely extracted while malic acid was still present in green beans. The temperature and pH did not seem to have any effect on the rate of diffusion for the two acids studied. However, the diffusivity constant of citric acid was higher than that of malic acid and the diffusion was faster for citric acid than for malic acid.

3.3.2. Folates

Folate diffusion took place in the first 60 min for spinach and 120 min for green beans, until reaching a plateau after 60 min for spinach and 120 min for green beans. Correlation coefficients exceeded 0.9 both for spinach and green beans. Results are presented in Table 1.

Folate diffusivity constants averaged at $7.45 \times 10^{-12} \text{ m}^2/\text{s}$ for spinach and $5.86 \times 10^{-10} \text{ m}^2/\text{s}$ for green beans. For green beans, the folate diffusivity constant was hundred times higher than for spinach meaning that the diffusion of folates is faster for green beans than for spinach. For spinach, neither temperature nor pH had an effect on the folate diffusivity constant. For green beans, the folate diffusivity constant appeared variable between batches for the same conditions. Indeed, the low slope of folate concentration decrease did not allow distinguishing a significant effect of pH or temperature on the folate diffusivity constant.

The diffusivity constants calculated for folates both from spinach and green beans were in the same order of magnitude than those calculated for acids and sugars from the same matrix.

4. Discussion

Our study was carried out at three temperatures (25, 45 and 65 °C), two of which were sufficient to allow thermal degradation of folates over the time scale used (45 and 65 °C). For diffusion kinetics, incubation at 85 °C proved to be impossible as the vegetables disintegrated over the time-course.

4.1. Diffusion of water soluble compounds

We could not follow folate, sugar and acid diffusion at 85 °C because of the matrix instability at this temperature. Indeed, at 85 °C for 4 h, spinach and green beans would have been completely disintegrated.

Overall, little data is currently available concerning water soluble molecule diffusion from plant tissues. The few studies available concern acid and sugar diffusion.

Vukov and Monzpart Senyi (1977) have determined diffusivity constants of sugars and acids from apple slice to water at 75 °C as 11.8×10^{-10} m²/s for sugars and 14.2×10^{-10} m²/s for acids.

Some experiments quantified diffusivity constants of glucose and sucrose from alginate gels to water, which are 2.55×10^{-10} at 5 °C for glucose and from 2.85×10^{-10} to 4.13×10^{-10} m²/s at 5 to 20 °C, for sucrose. From agar gels to water diffusivity constants are in the same range of magnitude at 2.47×10^{-10} m²/s at 5 °C (Friedman & Kraemer, 1930). Moreover, Schwartzberg and Chao (1982), determined diffusivity constants of glucose, fructose and sucrose to water at 25 °C from 0.69×10^{-9} to 0.54×10^{-9} m²/s.

Diffusivity constants calculated for acids and sugars in our study were in the range of 10^{-11} m²/s from spinach and 10^{-10} m²/s from green beans. From green beans, the constant we found is in agreement with those found in the literature for green beans (Friedman & Kraemer, 1930; Schwartzberg & Chao, 1982; Vukov & Monzpart Senyi, 1977). In contrast, diffusivity constants for spinach calculated in our study are lower than those found in the literature. However, the diffusivity constants of sugars and acids were calculated from alginate, agar gels or apple slices. These matrixes are different from those we studied, particularly the physiological barriers such as the cuticles which could explain the difference observed for spinach. For folates, currently no data is available in literature. The diffusivity constant calculated for folates are in the same order of magnitude as those calculated for sugars and acids, with the same difference between the two matrixes.

The system we have developed to study the diffusion of water-soluble molecules such as sugars, acids and folates from spinach and green beans appear to be efficient.

4.2. Impact of pH and temperature

Diffusivity constants calculated for folates from spinach and green beans, both at pH 7 and pH 5, were close to 10^{-12} and 10^{-10} m²/s respectively, and similar to the constants calculated for sugars and acids for both vegetables and at both pHs. The pH had no effect on folate, sugar and acid diffusion. At pH 5 and pH 7, the global negative electric charge of malic or citric acids, has the same polarity as that of cell walls. We can assume the existence of electrostatic repulsion between the cell walls and sugars and acids resulted in a limitation of diffusion. For green beans, a difference between the diffusivity constant of citric acid and malic acid of about a factor of ten was observed, although they are of similar size and charge in the conditions used. This difference could be due to the nature of the salts which neutralize citric acid, which have a greater ability to complex divalent cations. At pH 5 and pH 7, the overall electrical charge of folates is also negative (Zhao, Matherly,

& Goldman, 2009). Thus, it seems that the assumptions described for sugars and acids are transferable to folates, including the effects of electrostatic repulsion with cell walls. Moreover, folate interactions with macromolecules such as proteins could limit their diffusion under these pH conditions. Interaction between folates and proteins did not seem to be involved in our study because similar diffusivity constants were obtained for folates and sugars. The pH does not seem to be an important physico-chemical parameter for the diffusion of sugars, acids and folates.

Temperature did not have a significant effect on diffusion of water soluble compounds whatever matrix was considered, but had a significant effect on the residual ratio (C_{∞}/C_0) particularly for folates.

Residual levels of folates are lower for spinach (20% at pH 7 and 10–30% at pH 5) than for green beans (40–70% at pH 7 and 40–60% at pH 5). This indicates that more folates are extracted from spinach than from green beans. Spinach and green beans represent two different tissues, consisting of a leaf and pod respectively. Spinach leaves are composed of two layers, each with only a few cell layers. In contrast, green beans are composed of pods and seeds parenchyma, which are two different histological structures. The existence of these different compartments and the possibility that folates are retained in these compartments by bondage to macromolecules would result in a lower residual ratio and a slower rate of diffusion. In beans, the existence of a compartmentalization of folates in the parenchyma and in the seeds is clearly established. This was verified here: folate concentration in the parenchyma was 0.252 mg/kg while it was three times higher in the seeds with a concentration of 0.709 mg/kg.

4.3. Folate thermal degradation

Generally for each temperature, folate degradation kinetics were comparable in spinach and green beans. For all temperatures, a plateau was reached for both vegetables. The kinetics neither followed a first order nor a second order reaction, and a first order reaction with a partial conversion was still not satisfying, for the three temperatures and the two matrixes studied. This is in contradiction other studies where authors observed total folate degradation following a first order reaction (Mnkeni & Beveridge, 1983; Oey, Verlinde, Hendrickx, & Van Loey, 2006; Paine-Wilson & Chen, 1979).

Two effects could explain this phenomenon: the predominant loss was due to oxidation during thermal degradation and the second one was due to evolution of each individual derivative and their degradation products during the kinetics. Moreover, folate concentrations in samples from thermal degradation were determined by HPLC with fluorimetric detection after derivatization. This could lead to an artifact as it involved conversion of all derivatives to 5-CH₃-H₄folate. It could also convert degradation products formed during the kinetics into 5-CH₃-H₄folate, which would lead to an overestimation of the folate residual ratio.

4.4. Thermal degradation and industrial processing

Folate degradation observed in industrial processes (Delchier et al., 2013) appear low compared to the model systems which could be explained by the difference between the time considered during the experiments (4 h) and during processing (few minutes). Moreover, during sterilization of green beans, the can is a relatively small closed system, with less oxygen included than in our experiments. During this stage of the process, we observed a slight degradation of folates by about 10%. Moreover, the treatment time is 6–15 min, and therefore relatively short. During blanching, the system behaves similarly to the kinetics of thermal degradation that

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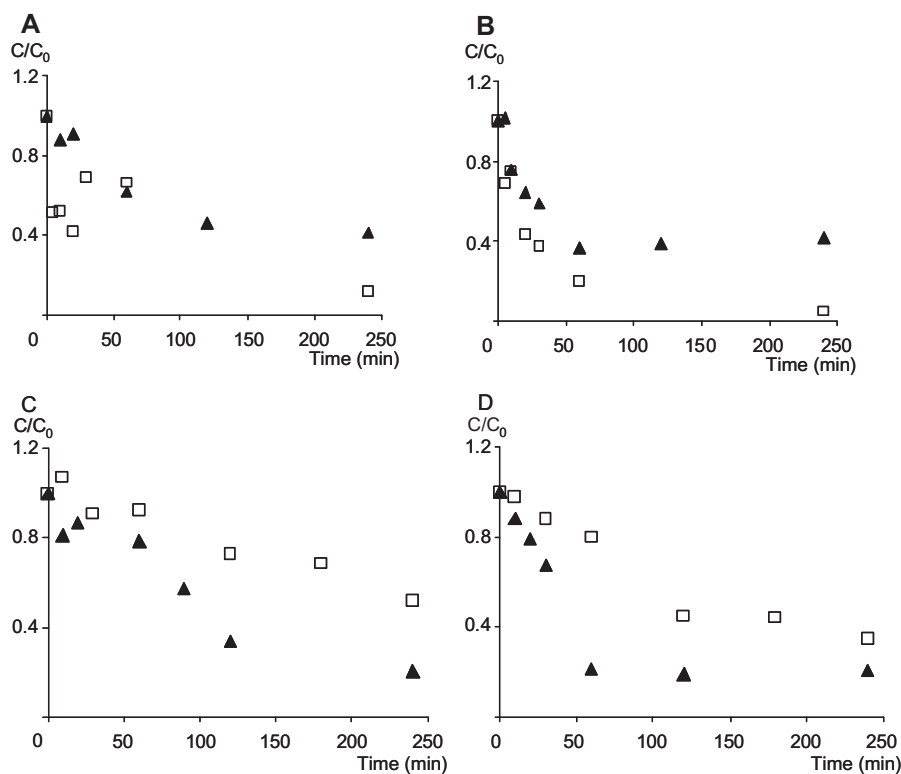


Fig. 3. Model of folate losses from spinach and green beans: diffusion vs. thermal degradation. Folate C/C_0 evolution over time both for diffusion + thermal degradation and for thermal degradation are represented by full triangle and empty squares respectively. On the top data for spinach at 45 °C (A) and 65 °C (B) are presented and on the bottom the respective data for green beans at 45 °C (C) and 65 °C (D).

we have performed here. During this stage, no losses of folates for both spinach and green beans were observed (Delchier et al., 2013). The blanching time applied in industrial processing is relatively short compared to those applied to the kinetics of thermal degradation (blanching of spinach: 70–120 s; green beans: 4–8 min) (Delchier et al., 2013). Moreover, the high temperatures used correspond to very low solubilized oxygen content (Winkler, 1888).

Finally, the matrices entering the blanching step are not disrupted yet. Thus, the results observed during industrial blanching are relatively consistent with the conclusions of the kinetics of thermal degradation.

4.5. Integrative model: impact of thermal degradation during diffusion kinetics

The thermal degradation kinetics conducted under the same oxygen conditions as the diffusion kinetics show residual folate amounts slightly higher at the end of the thermal degradation kinetics for spinach (diffusion: 35%, thermal degradation: 40%) (Fig. 3). In contrast to this, in green beans, the residual ratio (C_{∞}/C_0), measured at the end of the diffusion kinetics, are higher than those measured at the end of the thermal degradation kinetics (diffusion: 59% at 45 °C and 43% at 65 °C; thermal degradation: 20% for both temperatures). In addition, residual ratios are obtained with very different compositions. So it seems that the relative share of losses by diffusion and thermal degradation vary according to the vitamers considered. Moreover, the thermal degradation kinetics were performed on purees obtained from ground vegetables in cans, where grinding could play a role, either by facilitating the access of oxygen into puree or by modifying the local environment.

5. Conclusions

Our study enabled us to determine folate diffusivity constants, using a novel and efficient experimental device where neither pH nor temperature had a significant effect on the diffusivity constant, in contrast to the significant effect of the two vegetables matrices. Evolution of vitamers during diffusion was dependent on their nature. 5-CH₃-H₄folate and folic acid (PteGlu) were the two main derivatives lost during diffusion. These derivatives are more stable at pH 5 than at pH 7, so we can not exclude that these derivatives have been degraded over time, thus reducing the final concentration and inducing an increase in the total amount extracted. Evolution of derivatives during thermal degradation seems to be a key point for folate degradation by oxidation, which is why the next step of this study would be to determine the evolution of vitamers during oxidation in presence of different oxygen conditions.

By contrast, thermal degradation of folates in spinach and green beans was not monotonous especially at 45 °C and kinetics modelling by a first order with partial conversion was not satisfying. Comparison between diffusion and thermal degradation showed that folates were more sensitive to thermal degradation for green beans, while for spinach diffusion mechanism appears to be predominant over thermal degradation.

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