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Mechanisms of folate losses during processing: diffusion *vs.* heat degradation.

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Highlights:

1. pH and temperature have no effect on folate diffusivity constant

2. Vegetable matrices have effect on diffusivity constant

3. Folate stability during thermal treatment depend on their nature

Abstract:

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 conjointly 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 7.4×10^{-12} m^2/s for spinach and 5.8×10^{-10} m^2/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 vitamers 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

Introduction

Folate is the generic term used for different water-soluble vitamers 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). 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 of 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 to 80 % of initial folates and from 0 to 20 % in green beans (Klein, Lee, Reynolds & Wangles, 1979; Desouza & Eitenmiller, 1986; McKillop et al., 2002; Melse Boonstra et al., 2002; Delchier, Reich & Renard, 2012). However steaming and microwave cooking did not cause folate losses (Klein et al., 1979; McKillop et al., 2002; Delchier et al., 2012). 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 losses both during spinach freezing process and green beans canning process. Losses occur during the washing step for spinach and after sterilization for green beans, with folate found in the covering liquid (Delchier et al., 2013).

These 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 during spinach and green beans 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. Material 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 g/L 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, for facilitating stirring during 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 during 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 during 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 (Equation 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 (Equation 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 case of green beans, diffusivity constant was determined according to the cylinder solution given by Crank (1975) (Equation 3):

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

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 (Equation 4).

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

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

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

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 (Equation 6):

155 $$\ln\left(\frac{C}{C_0}\right) = -kt \quad (6)$$

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

158

159 **2.3. Analytical procedures**

160 **2.3.1. Folate measurement**

161 **2.3.1.1. Total folates content**

162 Total folate content was determined by HPLC with fluorimetric detection. After extraction all
163 folate vitamers were deconjugated into mono and diglutamate, reduced and methylated into 5-
164 CH_3-H_4folates. The latter were purified from the extract by affinity chromatography using
165 Folate Binding Protein, and quantified by RP-HPLC with fluorimetric detection (RF-1AXL,
166 Shimadzu inc., Kyoto, Japan). For experimental details, see Delchier et al. (2013).

167

168 **2.3.1.2. Stable isotope dilution assay**

169 Before extraction, labelled standards of folate vitamers were added and all folates were
170 deconjugated into their monoglutamate forms. Vitamers were purified on SPE SAX cartridges
171 after adding acetonitrile (10 mL) and centrifugation. Folate analysis was carried out on an
172 HPLC (Shimadzu inc., Kyoto, Japan) coupled with a triple quadrupole mass spectrometer (API
173 4000 Q-Trap, AB-Sciex, Foster City, CA, USA). A Pro-C18 HPLC-column (150 x 3, 3 µm,
174 130 Å, YMC, Japan) with water plus 0.1 % (v/v) formic acid (A) and acetonitrile plus 0.1 %
175 (v/v) formic acid (B) as mobile phases was used. The gradient started at 5 % B. After a linear
176 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 & Rychlik (2013).

2.3.2. 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), for malic acid (reference: 10.139.068), for glucose (reference: 10.716.251), for fructose (reference: 10.139.106) and for sucrose (reference: 10.716.251) were obtained from R-Biopharm (Darmstadt, Germany).

Sugars and acids were extracted from 200 mg of spinach or green beans 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 7400 g at 4 °C. 5 µL of supernatant were pipetted into the microplate. 250 µL of enzymatic reagents 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 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, 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, a plateau was reached with 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, folates 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-CH_3-H_4folate 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). For 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 ratio C_∞/C_0 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.

246 **3.2.2. Folate derivatives**

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

250 Both in spinach and green beans, the main compound was 5-CH_3-H_4folate which represented
251 about 70 % of folates at the beginning of diffusion and also revealed the highest loss of all
252 derivatives during diffusion. At pH 7, 5-CH_3-H_4folate residual ratios (C_∞/C_0) in spinach
253 decreased with increasing temperature. The same trend was observed with higher residual ratio
254 (C_∞/C_0) at pH 5. For green beans, 5-CH_3-H_4folate residual ratio (C_∞/C_0) decreased with
255 temperature increase at pH 7 and pH 5.

256 The second main class of folates derivatives was formyl derivatives. 10-HCO-PteGlu and 5-
257 HCO-H_4folate represented 6 to 16 % in spinach and 7 to 12 % in green beans respectively. 5-
258 HCO-H_4folate residual ratios (C_∞/C_0) increased with the temperature increase both in spinach
259 and green beans between pH 7 and pH 5. 10-HCO-PteGlu residual ratios (C_∞/C_0) were quite
260 stable at 25 °C both at pH 7 and 5 while it increased with rising temperature both at pH 7 and 5.

261 Residual ratios (C_∞/C_0) of minor derivatives (PteGlu, H_4folate, 5,10-CH^+-H_4folate and
262 10-HCO-H_2folate) were very low and appeared quite variable during kinetics, both for spinach
263 and green beans.

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

269

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 corresponding 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 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 °C 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. Both for glucose and malic acid, temperature and pH did not have an effect on residual ratios.

For both pH and temperature the concentration in sugars and green beans 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 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.

Both for 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 5.5×10^{-12} m^2/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 bean, which was on average 6.1×10^{-10} m^2/s

Diffusion of acids (citric and malic acids) from green beans showed diffusivity constants of the same order for both acids at all temperatures and pH, on average about 4.6×10^{-10} m^2/s (Table

318 1). However, plateau levels were very different. Citric acid was almost completely extracted
319 while malic acid was still present in green beans. The temperature and pH did not seem to have
320 any effect on the rate of diffusion for the two acids studied. However, the diffusivity constant
321 of citric acid was higher than that of malic acid and the diffusion was faster for citric acid than
322 for malic acid.

323

324 **3.3.2. Folates**

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

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

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

337 **4. Discussion**

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

342

343 **4.1. Diffusion of water soluble compounds**

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

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

349 Vukov & Monszpart Senyi (1977) have determined diffusivity constants of sugars and acids
350 from apple slice to water at 75 °C as 11.8×10^{-10} m^2/s for sugars and 14.2×10^{-10} m^2/s for acids.

351 Some experiments quantified diffusivity constants of glucose and sucrose from alginate gels to
352 water, which are 2.55×10^{-10} at 5 °C for glucose and from 2.85×10^{-10} m^2/s to 4.13×10^{-10} m^2/s at
353 5 °C to 20 °C, for sucrose. From agar gels to water diffusivity constants are in the same range
354 of magnitude at 2.47×10^{-10} m^2/s at 5 °C (Friedman & Kraemer, 1930). Moreover, Schwartzberg
355 & Chao (1982), determined diffusivity constants of glucose, fructose and sucrose to water at 25
356 °C from 0.69×10^{-9} to 0.54×10^{-9} m^2/s.

357 Diffusivity constants calculated for acids and sugars in our study were in the range of 10^{-11} m^2/s
358 from spinach and 10^{-10} m^2/s from green beans. From green beans, the constant we found is in
359 agreement with those found in the literature for green beans (Friedman & Kraemer, 1930;
360 Vukov & Monszpart Senyi, 1977; Schwartzberg & Chao, 1982). In contrast, diffusivity
361 constants from spinach calculated in our study are lower than those found in the literature.
362 However, the diffusivity constants of sugars and acids were calculated from alginate, agar gels
363 or apple slices. These matrixes are different from those we studied, particularly the
364 physiological barriers such as the cuticles which could explain the difference observed for
365 spinach. For folates, currently no data are available in literature. The diffusivity constant

366 calculated for folates are in the same order of magnitude as those calculated for sugars and
367 acids, with the same difference between the two matrices.
368 The system we have developed to study the diffusion of water-soluble molecules such as
369 sugars, acids and folates from spinach and green beans appear to be efficient.
370
371 **4.2. Impact of pH and temperature**
372 Diffusivity constants calculated for folates from spinach and green beans, both at pH 7 and pH
373 5, were close to 10^{-12} m^2/s and 10^{-10} m^2/s respectively, and similar were the constants calculated
374 for sugars and acids for both vegetables and at both pH. The pH had no effect on folate, sugar
375 and acid diffusion. At pH 5 and pH 7, the global negative electric charge of malic or citric
376 acids, has the same polarity as that of cell walls. We can assume the existence of electrostatic
377 repulsion between the cell walls and sugars and acids resulting in a limitation of diffusion. For
378 green beans, a difference between the diffusivity constant of citric acid and malic acid of about
379 a factor of ten was observed, although they are of similar size and charge in the conditions
380 used. This difference could be due to the nature of the salts which neutralize citric acid which
381 has a greater ability to complex divalent cations. At pH 5 and pH 7, the overall electrical
382 charge of folates is also negative (Zhao, Matherly & Goldman, 2009). Thus, it seems that the
383 assumptions described for sugars and acids are transposable to folates, including the effects of
384 electrostatic repulsion with cell walls. Moreover, folate interactions with macromolecules such
385 as proteins could limit their diffusion under these pH conditions. Interaction between folates
386 and proteins did not seem to be involved in our study because similar diffusivity constant were
387 obtained for folates and sugars. The pH does not seem to be an important physico-chemical
388 parameter for the diffusion of sugars, acids and folates.

389 Temperature did not have a significant effect on diffusion of water soluble compounds
390 whatever matrix was considered but had a significant effect on the residual ratio (C_∞/C_0)
391 particularly for folates.
392 Residual levels of folates are lower for spinach (20 % at pH 7 and 10 to 30 % at pH 5) than for
393 green beans (40 to 70 % at pH 7 and 40 to 60 % at pH 5). This indicates that folates are more
394 extracted from spinach than from green beans. Spinach and green beans represent two different
395 tissues, consisting of leaf and pod respectively. Spinach leaves are composed of two layers,
396 each with only a few cell layers. In contrast, green beans are composed of pods and seeds
397 parenchyma, which are two different histological structures. The existence of these different
398 compartments and the possibility that folates are retained in these compartments by bondage to
399 macromolecules would result in a lower residual ratio and a slower rate of diffusion. In beans,
400 the existence of a compartmentalization of folates in the parenchyma and in the seeds is clearly
401 established. This was verified here: folate concentration in the parenchyma was 0.252 mg/kg
402 while it was three times higher in the seeds with a concentration of 0.709 mg/kg.
403
404 **4.3. Folate thermal degradation**
405 Generally for each temperature, folate degradation kinetics were comparable in spinach and
406 green beans. For all temperatures, a plateau was reached for both vegetables. The kinetics
407 neither followed a first order nor a second order reaction, and a first order reaction with a
408 partial conversion was still not satisfying, for the three temperatures and the two matrixes
409 studied. This is in contradiction other studies where authors observed total folate degradation
410 following a first order reaction (Paine-Wilson & Chen, 1979; Mnkeni & Beveridge, 1983; Oey,
411 Verlinde, Hendrickx & Van Loey, 2006).
412 Two effects could explain this phenomenon: the predominant loss was due to oxidation during
413 thermal degradation and the second one was due to evolution of each individual derivative and

414 their degradation products during the kinetics. Moreover, folate concentrations in samples from
415 thermal degradation were determined by HPLC with fluorimetric detection after derivatization.
416 This could lead to an artifact as it involved conversion of all derivatives to 5-CH_3-H_4folate. It
417 could also convert degradation products formed during the kinetics into 5-CH_3-H_4folate, which
418 would lead to an overestimation of the folate residual ratio.
419

420 **4.4. Thermal degradation and industrial processing**

421 Folate degradation observed in industrial processes (Delchier et al., 2013) appear low
422 compared to the model systems which could be explained by the difference between the time
423 considered during the experiments (4 hours) and during processing (few minutes). Moreover,
424 during sterilization of green beans, the can is a relatively small closed system, with less oxygen
425 included than in our experiments. During this stage of the process, we observed a slight
426 degradation of folates by about 10 %. Moreover, the treatment time is 6-15 min, and therefore
427 relatively short. During blanching, the system behaves similarly to the kinetics of thermal
428 degradation that we have performed here. During this stage, no losses of folates for both
429 spinach and green beans were observed (Delchier et al., 2013). The blanching time applied in
430 industrial processing is relatively short compared to those applied to the kinetics of thermal
431 degradation (blanching of spinach: 70 s to 120 s; green beans: 4-8 min) (Delchier et al., 2013).
432 Moreover, the high temperatures used correspond to very low solubilized oxygen content
433 (Winkler, 1888).
434 Finally, the matrices entering blanching step are not disrupted yet. Thus, the results observed
435 during industrial blanching are relatively consistent with the conclusions of the kinetics of
436 thermal degradation.
437

438 **4.5. Integrative model: impact of thermal degradation during diffusion kinetics**

439 The thermal degradation kinetics conducted under the same oxygen conditions as the diffusion
440 kinetics show residual folate amounts slightly higher at the end of the thermal degradation
441 kinetics for spinach (diffusion: 35 %, thermal degradation: 40 %) (Fig. 3). In contrast to this, in
442 green beans, the residual ratio (C_∞/C_0) measured at the end of the diffusion kinetics are higher
443 than those measured at the end of the thermal degradation kinetics (diffusion: 59 % at 45 °C
444 and 43 % at 65 °C; thermal degradation: 20 % for both temperatures). In addition, residual
445 ratios are obtained with very different compositions. So it seems that the relative shares of
446 losses by diffusion and thermal degradation vary according to the vitamers considered.
447 Moreover, the thermal degradation kinetics were performed on purees obtained from ground
448 vegetables in cans, where grinding could play a role, either by facilitating the access of oxygen
449 into puree or by modifying the local environment.
450
451 **Conclusion**
452 Our study enabled us to determine folate diffusivity constants, using a novel and efficient
453 experimental device where neither pH nor temperature had a significant effect on the
454 diffusivity constant, in contrast to the significant effect of the two vegetables matrices.
455 Evolution of vitamers during diffusion was dependent on their nature. 5-CH_3-H_4folate and folic
456 acid (PteGlu) were the two main derivatives lost during diffusion. These derivatives are more
457 stable at pH 5 than at pH 7, so we can not exclude that these derivatives have been degraded
458 over time, thus reducing the final concentration and inducing an increase in the total amount
459 extracted. Evolution of derivatives during thermal degradation seems to be a key point for
460 folate degradation by oxidation, which is why a next step of this study would be to determine
461 the evolution of vitamers during oxidation in presence of different oxygen conditions.
462 By contrast, thermal degradation of folates in spinach and green beans was not monotonous
463 especially at 45 °C and kinetics modelling by a first order with partial conversion was not

satisfying yet. 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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529

Figure Captions

Fig. 1. Folates' thermal degradation in spinach and green beans purees

The left part of the graphic 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 graphic represent 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.

Fig. 2. Folate diffusion from spinach and green beans

On the left graphic (A) experimental and modelling data for folate diffusion from spinach at pH 7 and 45 °C are presented and on the right graphic (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-CH_3-H_4folate monoglutamate and represented by full lozenges. Concentrations determined by second Fick's law model are represented by the dotted line. Right graph (A)

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).

Fig.1. Delchier et al.

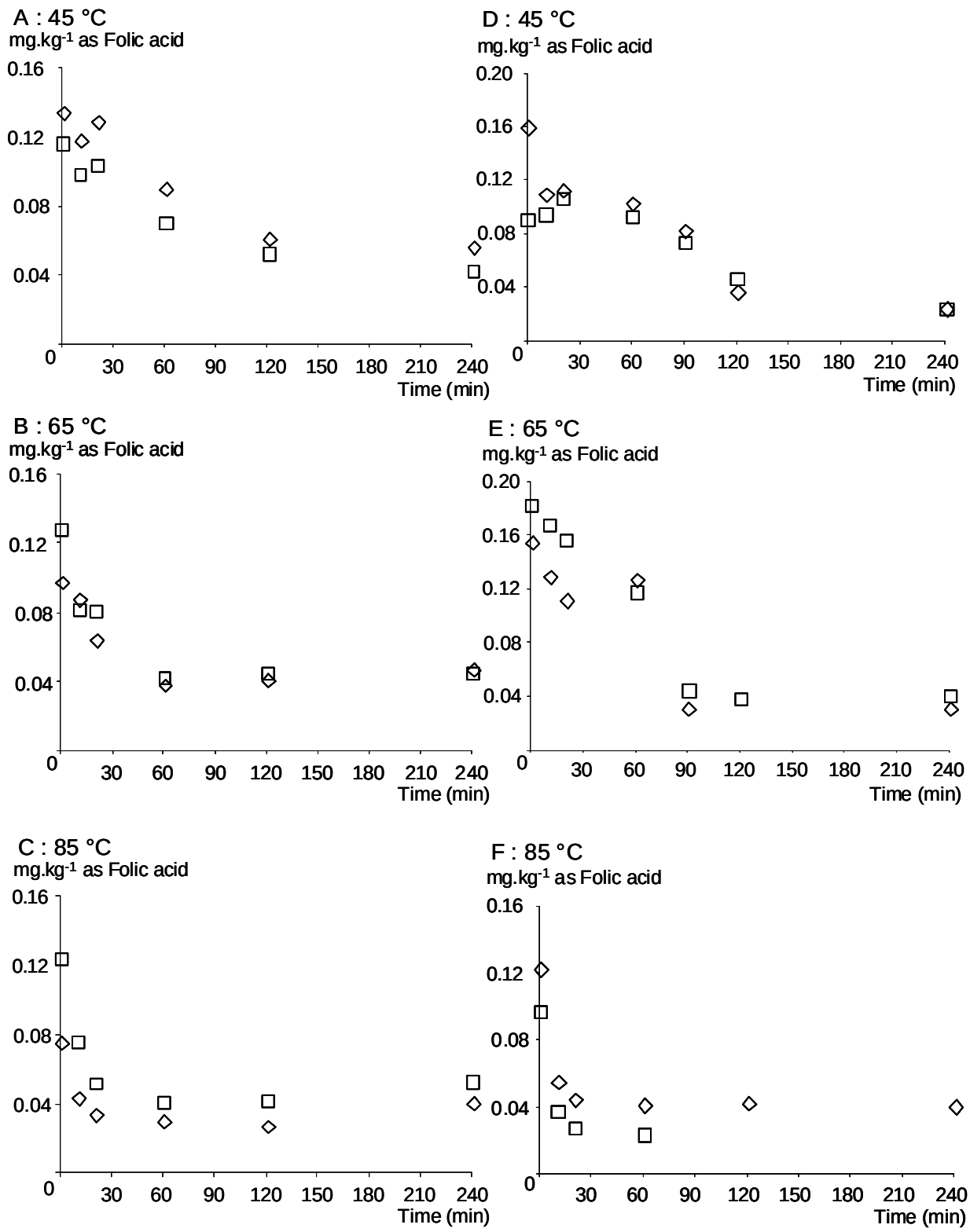
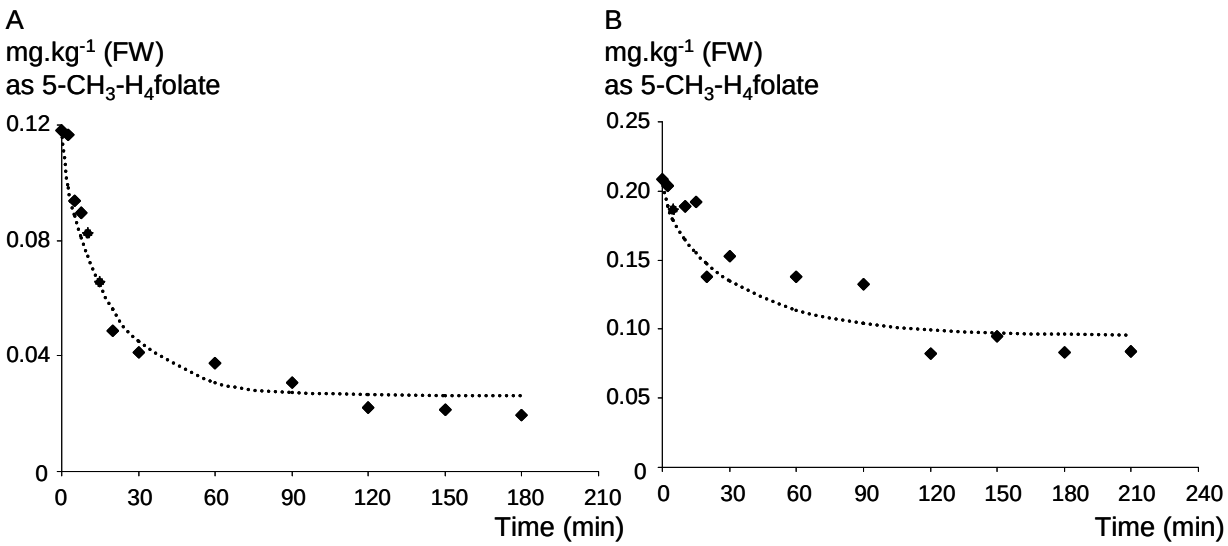


Fig.2. Delchier et al.



613 Fig.3. Delchier et al.

614

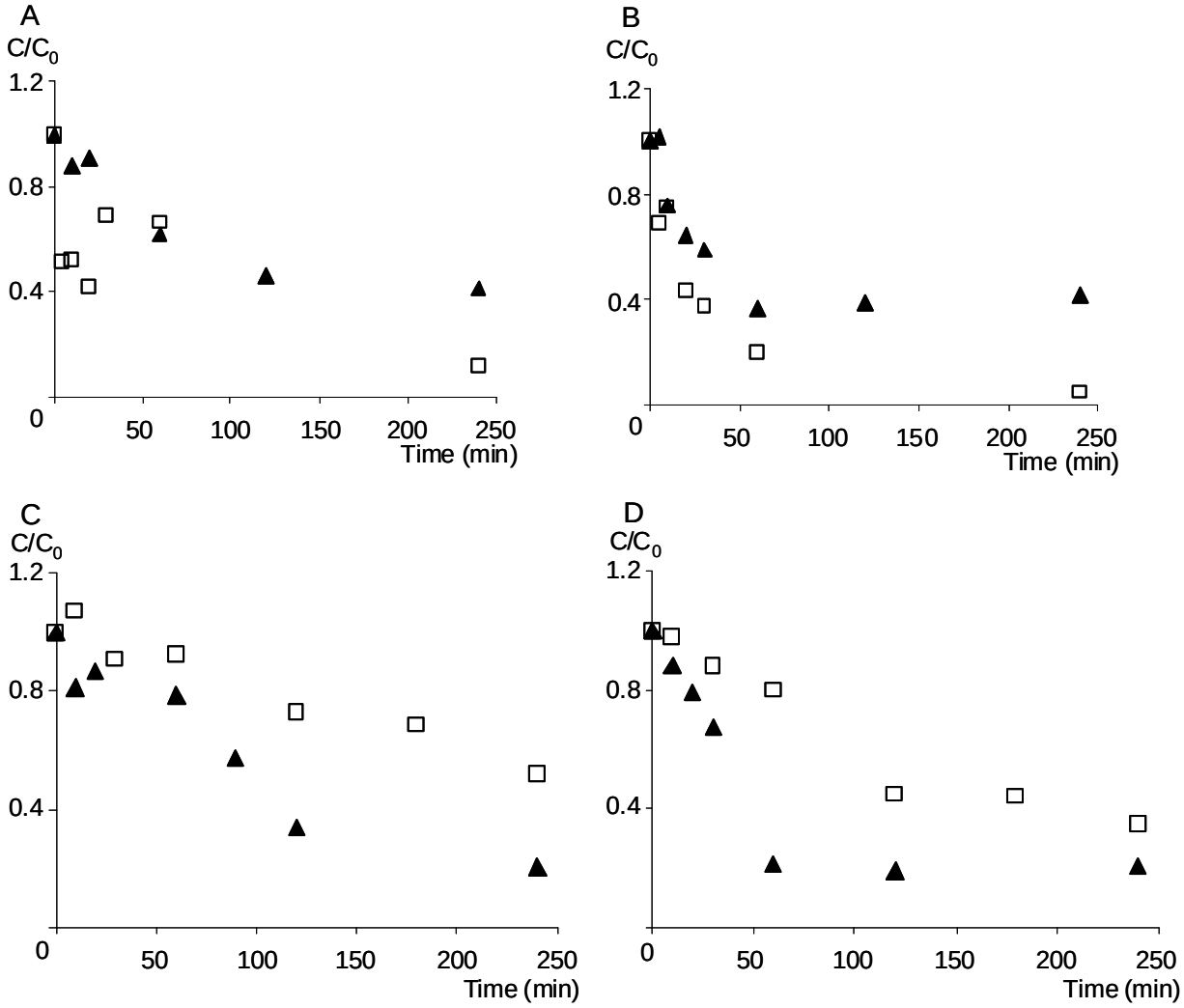


Table 1: Diffusivity and C/C_0 of folates, sugars and acids in spinach and green beans

			Folates		Glucose		Fructose		Sucrose		Malic acid		Citric acid	
	pH	T	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	$7 \pm 2.1 .10^{-12}$	0,28 ± 0.07	$3.0.10^{-12}$	0.50	-	-	-	-	$3,5.10^{-12}$	0,49	-	-
		45	$7 \pm 1.7.10^{-12}$	0,23 ± 0.03	$1.0.10^{-11}$	0.30	-	-	-	-	$6,0.10^{-12}$	0,19	-	-
		65	$6 \pm 1.1.10^{-12}$	0,28 ± 0.12	-	0.86	-	-	-	-	$2,5.10^{-11}$	0,52	-	-
	5	25	$6 \pm 2.1.10^{-12}$	0,33 ± 0.01	$6,0.10^{-12}$	0,54	-	-	-	-	$3,5.10^{-12}$	0,57	-	-
		45	9	0,38 ± 0.14	$8,0.10^{-12}$	0,02	-	-	-	-	$5,5.10^{-12}$	0,55	-	-
		65	$8 \pm 1.4.10^{-12}$	0,12 ± 0.04	$9,5.10^{-12}$	0,27	-	-	-	-	$2,5.10^{-11}$	0,27	-	-
Green beans	7	25	$7 \pm 1.1.10^{-10}$	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	$8 \pm 1.7.10^{-10}$	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	$6 \pm 2.0.10^{-10}$	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	$5 \pm 1.4.10^{-10}$	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	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 calculate for folates, Glucose, fructose, Sucrose, Malic acid and Citric acid in $m^2.s^{-1}$. For folates diffusivity constant, results are expressed as mean ± standard deviation.

C/C_0 corresponds to the residual concentration calculate for folates, Glucose, fructose, Sucrose, Malic acid and Citric acid. For folates, C/C_0 is expressed as mean ± standard deviation.

Diffusivity constant for folates was calculated from concentrations expressed as 5-CH_3-H_4folate monoglutamate (after conversion of all derivatives)

T corresponds to the temperature in degree Celsius.

615 Table 2: Residual concentration of folate derivatives in spinach during diffusion kinetics

pH	°C	Time	5-CH_3-H_4folate	5-HCO-H_4folate	10-HCO-PteGlu	H_4folate	PteGlu	5,10-CH^+-H_4folate	10-HCO-H_2folate	Total folates (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*	*ø*	*0,31*	*ø*	*ø*	*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*

616 Results are expressed in mg kg^{-1} of fresh weight, in blanched spinach (t_0) and blanched
617 spinach after diffusion (t_{180} or t_{240}).
618 C/C_0 is the ratio between the initial concentration and the final concentration.
619 °C corresponds to the temperature in degree Celsius.
620
621
622

623 Table 3: Residual concentration of folate derivatives in green beans during diffusion kinetics
624
625

pH	°C	Time	5-CH_3-H_4folate	5-HCO-H_4folate	10-HCO-PteGlu	H_4folate	PteGlu	5,10-CH^+-H_4folate	10-HCO-H_2folate	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
		0,43	*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*

626
627 Results are expressed in mg kg^{-1} of fresh weight, in blanched green beans (t_0) and blanched
628 green beans after diffusion (t_{180} or t_{240}).
629 C/C_0 is the ratio between the initial concentration and the final concentration.
630 °C corresponds to the temperature in degree Celsius.
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