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Degradation of folic acid in fortified vitamin juices during long term storage

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Abstract
Folic acid (FA) concentrations of nine fortified vitamin juices were determined with the aim to study the FA degradation and to investigate the deviation from the declared label value. The juices were received shortly after bottling and were analyzed monthly during controlled storage conditions (light and dark) over one year. The analyses were performed by HPLC–MS/MS, which included a fast "dilute and shoot" sample preparation. Average decreases in FA concentration of 46% were observed after one year. Fresh juices (shortly after bottling) showed the highest deviations from the declared label value (up to + 89%). Label values did not reflect the actual concentration of FA in these products, making it difficult to determine the intake of this vitamin.

Keywords: Folic acid, Fortified vitamin juices, HPLC-MS/MS

1. Introduction

The generic term "folates" refers to a group of naturally occurring B-vitamins. They share a similar chemical structure and as being essential vitamins, folates must be obtained from dietary sources. Previous studies have shown a low intake of folates in Germany and the most European countries (Elmadfa, 2009; Krems, Walter, Heuer, & Hoffmann, 2012). Pteroyl-L-monoglutamic acid (FA) a synthetic form of folate, is approved by the EC regulation No. 1925/2006 (European Parliament, 2006) for the fortification of food to enable an adequate vitamin intake. This regulation does not specify a maximum fortification amount of FA in food products. Folates are known to be sensitive to heat, oxygen, light and low pH (Akhtar, Khan, & Ahmad, 1999; McKillop et al., 2002). Degradation occurs during storage. This is especially true for vitamin juices, mixtures of different fruit juices fortified with several vitamins, where a low pH (3.5) prevails. Producers of vitamin juices declare the concentration of fortified FA on the label as the sum of natural and added amount and guarantee for that amount until the expiration date. In order to do so they need to add a higher amount when producing the fresh juices to compensate for the loss that occurs during storage.

At this point there is no European mandatory regulation as to how far the added amount of vitamins may exceed the declared concentration on the label. There are recommended practices on national bases like a position paper worked out by the German Food Chemical Society (LChG) regarding recommendations concerning the tolerance of nutrient declarations (LChG, 2009). For vitamins a tolerance of ±30% for FA compared to the label declaration is generally accepted. Due to instability of some vitamins an additional dosage of up to 50% is considered necessary and may even be exceeded in some cases, like the fortification of fruit juices.

Most recently the EU commission has provided a draft on guiding principles with reference to the setting of tolerances for nutrient values (European Commission, 2012). According to this draft the acceptable deviation of the declared label value regarding vitamins is +50%. So far this draft has no legal status but acknowledges the need for a regulation in this matter.

Due to the mentioned instability and degradation of vitamins in fruit juices, we expected producers to add significantly higher concentrations of vitamins to ensure the labeled value until the expiration date. We were particularly interested in the concentration of added FA as most recent studies have pointed out some possible ambivalent health effects. While an adequate folate intake is beneficial during pregnancy to prevent congenital malformations such as neural tube defects (Scholl & Johnson, 2000), adverse

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effects of a high FA intake are in discussion. An intake above the tolerable upper intake level of 1 mg/day FA (EFSA, 2006) can mask a vitamin B12 deficiency, which untreated will lead to neurological damages (Clarke et al., 2003). New studies also suggest that a high FA intake accelerates the malignant transformation of existing neoplasms (Kim, 2007a,b; Stolzenberg-Solomon et al., 2006) and reduces the efficacy of antifolate drugs used for instance for the treatment of rheumatoid arthritis or psoriasis (Khanna et al., 2005; Salim, Tan, Ilchyshyn, & Berth-Jones, 2006).

Our aim was to investigate the degradation of FA during the storage duration of twelve months and to compare the producers' declaration on the label with the concentrations found in the juices. We also studied the influence of light on the degradation process. We opted for a simple and quick yet accurate and precise method to analyze the juices.

2. Experimental/materials and methods

2.1. Reagents and materials

FA was purchased from Sigma Aldrich (Deisenhofen, Germany). Fivefold isotope-labeled FA (C13-FA) as internal reference substance (IS) was purchased from Merck&Cie (Schaffhausen, Switzerland). The purity of the substance was 99.7% with an isotope-labeling exclusively on the glutamate part of the molecule. Acetonitrile (HPLC grade), formic acid (99%) and sodium hydroxide were obtained from VWR (Darmstadt, Germany), 2-(cyclohexylamino) ethanesulfonic acid (CHES), 4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid (HEPES) and dithiothreitol (DTT) were from Sigma Aldrich (Deisenhofen, Germany). Analytical grade water was obtained from a LaboStar TM purification system, Siemens (Munich, Germany). Methanol was from Merck (Darmstadt, Germany). Phenex-PTFE syringe filters (15 mm, 0.2]m) were obtained from Phenomenex (Aschaffenburg, Germany).

A HEPES/CHES buffer according to Wilson and Horne (Wilson & Horne, 1984) was used for diluting FA stock solutions and juice samples. Stock solutions of the FA standard and the IS (2 mg/ml) were prepared in an aqueous solution of NaOH (0.1 mol/l). These solutions were diluted with HEPES/CHES buffer and stored in small portions at -80 °C until use. The working solution of the IS was 1 µg/ml, various concentration of the FA standard were prepared by appropriate dilution with the HEPES/CHES buffer.

2.2. HPLC-MS conditions

HPLC analyses were carried out using an Agilent 1200 series HPLC system (Agilent, Waldbronn, Germany). The system comprised a vacuum degasser, binary pumps, a thermostated autosampler and column compartment. The column used for the chromatography was a Prontosil C-18 (Bischoff, 3 × 150 mm, 3]m) at a flow rate of 0.5 ml/min and a column temperature of 35 °C. Injection volume was 10]l. The HPLC mobile phases consisted of 0.1% aqueous formic acid (A) and acetonitrile (B). Following linear gradient was used (%B): 0–5 min (5%), 15 min (28%), and 16–20 min (100%). The column was then re-equilibrated for 10 min, making a total run time of 30 min. A switching valve was used in order to protect the instrument from unnecessary matrix pollution. The HPLC eluent flow between 12 and 18 min was directed into the mass spectrometer while the rest of the run went to waste. The FA signal was detected at 15 min.

The HPLC was coupled to a 3200 QTrap mass spectrometer (AB Sciex, Darmstadt, Germany). Ionization was achieved using positive electrospray ionization. Following conditions were found to be optimal: 21 V (DP), 20 psi (CUR), 450 °C (Source Temperature), 5000 V (Ion Spray Voltage), 55 psi/60 psi (Ion Gas 1 and 2, respectively). Data were recorded in the multiple reactions monitoring (MRM) mode using following transitions: FA 442.1 > 295.3 (CE 21 V), 442.1 > 176.2 (CE 53 V), C13-FA 447.1 > 295.3 (CE 23 V), 447.1 > 176.2 (CE 53 V). All aspects of system operation and data acquisition were controlled using Analyst 1.5.2 software (AB Sciex).

2.3. Sample preparation

Juice samples, matrix calibration samples and quality control (QC) samples were prepared equally: 1 ml juice was mixed with 1 ml 0.1% aqueous NaOH and 100 µl IS working solution. The samples were diluted with 2 ml HEPES/CHES buffer, homogenized and centrifuged. The supernatant was filtered through a syringe filter prior to analysis.

2.4. Quantitation and method validation

Quantitation of the FA concentration was done by using the peak area ratios of unlabeled to labeled compound using a matrix calibration curve. This curve was prepared with a non-fortified vitamin juice spiked in the following concentration levels: 50, 100, 200, and 400 µg/100 ml of FA.

Comparison of aqueous standard samples with matrix samples showed a process efficiency of 90% (±11%) (Matuszewski, Constanzer, & Chavez-Eng, 2003). The use of the IS compensated for the matrix effect.

A fortified vitamin juice was used as QC sample for each sample batch. One batch consisted of the packages of one juice throughout the entire year (13 sampling points with 3 individual packages). Our QC sample had been analyzed by three independent laboratories prior to our analyses. Measured mean concentrations of the QC samples ($M = 146$ µg/100 ml) were in good accordance to the results from the other laboratories (data of the inter-laboratory comparison can be found in the Supplemental). QC sample results of each batch were monitored via an individual moving range control chart (IMR-Chart). The precision of the QC samples over the range of the entire study ($n = 16$) was 6.5% (CV).

2.5. Juice samples

Samples (original packages) of nine commonly sold vitamin juices (juices from well-known brands as well as discounter products) were delivered from several producers in Germany right after the filling to the Max Rubner-Institut. Upon arrival the juices were stored in a dark room at 1 °C until the first sampling which marks the beginning of the study. We refer to the first sampling as the initial analyses. The packaging of the juices varied: glass bottles (brown and clear), PET (brown and clear) and cardboard boxes. The labeled FA concentration was 100 µg/100 ml, except for one juice with 60 µg/100 ml. A list of the vitamin juices with data of their packaging type, storage condition, label and storage time after filling can be found in Table 1.

2.6. Long term storage experiment

The study and therefore the initial analyses of the juices started as soon as all juices were available, which was between 11 and 32 days after filling by the producer.

The vitamin juices were stored for the duration of one year under controlled conditions. Samples from all juices ($n = 9$) were stored in the dark at 18 °C. Six of these came in light-transmissive packaging. Samples of these six juices were additionally stored under the influence of light (18 °C, 500 Lux for 10 h/day). These storage parameters were chosen to reflect common storage conditions, e.g., at a supermarket. Three independent juice packages

Table 1
Data summary of the analyzed juices ($n = 9$).

Juice number and storage condition		Packaging	FA concentration label μg/100 ml	Storage time before initial sampling (days)
1	Dark	PET brown	100	32
	Light			
2	Dark	PET brown	100	20
	Light			
3	Dark	Glass clear	100	28
	Light			
4	Dark	Glass clear	100	20
	Light			
5	Dark	PET clear	100	16
	Light			
6	Dark	PET clear	100	15
	Light			
7	Dark	Cardboard	100	12
8	Dark	Cardboard	60	11
9	Dark	Cardboard	100	28

were drawn monthly from each juice respectively and analyzed for the determination of FA.

2.7. Statistical approach

2.7.1. General

We used a nonlinear mixed model to determine the relationship between FA concentration and the factors storage time and storage condition (light and dark storage). The formulas describing this nonlinear relationship are derived from a first order reaction in its integral form with a slight modification: an additional constant was added to the term because omitting this additive constant lead to poor model fit. Random effects were added to the exponential decay model of FA for all parameters to account for juice specific differences. Time was considered as a continuous variable.

Further, we observed that variances between the sampling time points (months) were unequal. Therefore, we adapted the variance structure of the model. Whether there was an improvement in model fit by using this modified variance structure was assessed by a likelihood ratio test. If the likelihood ratio test reached a significance level of 0.05, the model with the modified variance structure was used for further analysis.

Regression assumptions were approved by visual inspection of the residual versus fitted plot (homoscedasticity) and QQ-plots in order to check normal distribution of residuals. All calculations were carried out by R 2.15.0 (R Development Core Team, 2012) and the R package *nlme* 3.1-103 (Pinheiro, Bates, DebRoy, Sarkar, & R Core Team, 2012) for estimation of the described models below.

2.7.2. Degradation dark storage

To describe the degradation of those juices ($n = 9$) stored in the dark "model equation I" was used:

$$folic = \beta_0 + \beta_1 \cdot e^{-\beta_2 t} \quad \text{(model equation I)}$$

β_0 is the intercept, β_1 is the initial value of the exponential part, β_2 stands for the decay rate of the exponential function for the dark storage condition. Time t was measured in months, *folic* is the FA concentration in $\frac{\mu g}{100ml}$.

3.1.1. Influence of light during storage

To investigate the effect of light "model equation II" was used ($n = 6$ juices, stored under light and dark conditions respectively) with the same parameterization as above with an additional dummy variable (*storage*).

$$folic = \beta b_0 + \beta_1 \cdot e^{-(\beta_2 + \beta_3 \cdot storage)t} \quad \text{(model equation II)}$$

This variable *storage* takes on the value 1 for the light storage condition and 0 respectively for the dark storage condition. Thus the decay rate in the light storage condition is defined by the sum of β_2 and β_3, while for the dark storage condition the decay rate is solely expressed by β_2. Hence, β_3 can be interpreted as the difference in the decay rate between light and dark storage condition.

To determine how far the measured concentrations deviate from the label value, we first calculated the deviation for each individual juice in % to their respective label value. We then used the arithmetic mean (*M*) of the results to determine an average deviation from the label values.

3. Results

The initial average vitamin concentration found in all juices stored in the dark were 176 μg/100 ml (ranging from 135–245 μg/100 ml, $n = 9$) for FA. After one year of storage the average concentration in these juices was 95 μg/100 ml (ranging from 65–129 μg/100 ml) for FA. These data are presented as a box plot gra phic to display the time-dependent degradation process (Fig. 1). The parameter estimates for "model equation I" describing the degradation can be found in Table 2.

The juices showed a significant decrease in FA concentration, represented by β_2 (p-value < 0.001) with an average loss of 81 μg/100 ml (46%) over the course of the entire study. Examining the relationship between FA concentration and time dimension it was evident that degradation of FA was stronger in the beginning. The curve flattens at the end of the measured period.

To investigate the effect of light we looked at the six juices that came in light-transmissive packages and the respective data from the two different storage conditions. The exponential decay model "model equation II" which describes the degradation of FA in these six juices is presented in Fig. 2 and parameter estimates are given in Table 3.

The parameters showed that the degradation rate was significantly higher under the influence of light (β_3, $p = 0.024$.): These juices were found to have an average of 6 μg/100 ml less FA after one year of storage. A summary of the measured concentrations is given in Table 4.

We determined how far the actual FA concentrations differ from the label value (Fig. 3) and observed the highest deviation in the fresh (initial) juices (+89% dark, $n = 9$).

The expiration date of the juices ranged from 6–12 months after the filling date. After six months of storage the deviations from the label were (+24% dark, $n = 9$; +14% with light, $n = 6$). After twelve

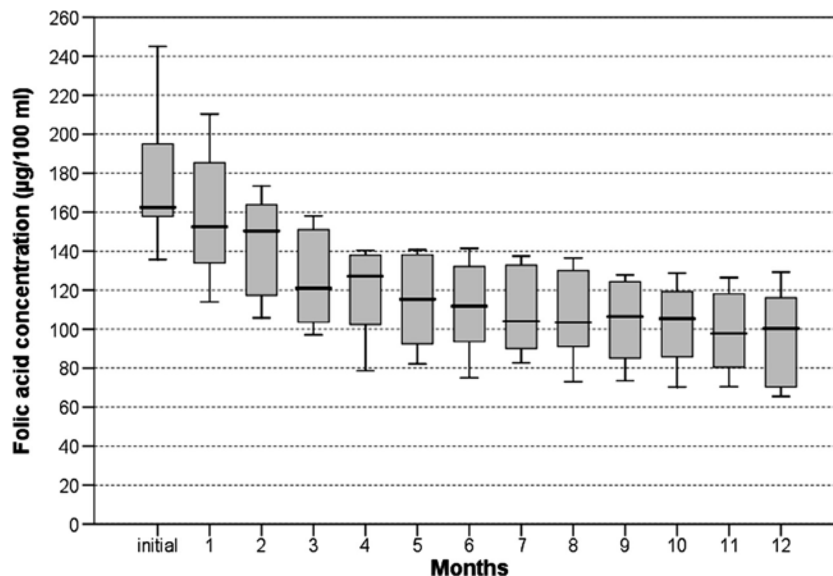

Fig. 1. Box plots showing the monthly FA concentrations (µg/100 ml) of juices stored in the dark (n = 9): the box portion of the box plot includes 50% of the data from the 25th to the 75th percentile while the whiskers extend to the minimum and maximum data values (SPSS version 20.0, IBM Corporation, Armonk, New York, USA).

Table 2
Parameter estimates corresponding to "model equation I".

Parameter	Value	Std. error	Degrees of freedom	p-Value
β_0	96.686	6.9507	315	<0.0001
β_1	78.218	7.3764	315	<0.0001
β_2	0.281	0.0132	315	<0.0001

Table 3
Parameter estimates corresponding to "model equation II".

Parameter	Value	Std. error	Degrees of freedom	p-Value
β_0	88.882	8.371	442	<0.0001
β_1	92.932	10.014	442	<0.0001
β_2	0.191	0.0187	442	<0.0001
β_3	0.0790	0.0350	442	0.0244

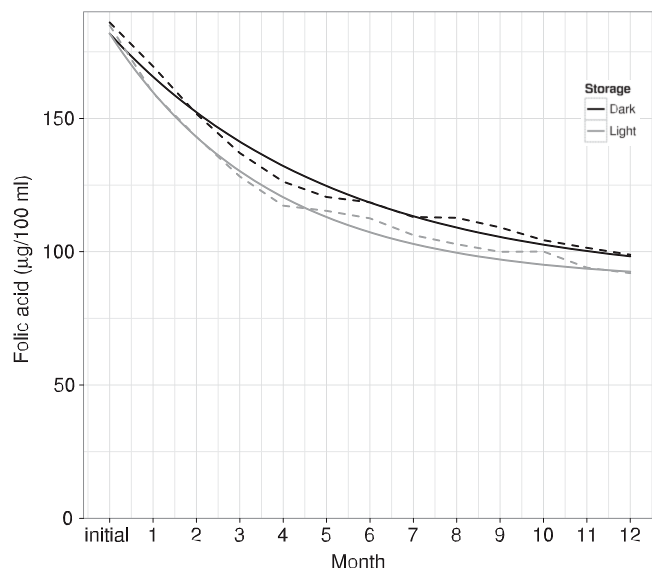

Fig. 2. Non-linear mixed model of FA degradation process: Juices stored under the influence of light (L, n = 6) and stored in the dark (D, n = 6). While the dotted lines represent the measured data of the juices (average pooled by month), the continuous lines reflect the prediction of the non-linear mixed model approach.

Table 4
Mean (M) FA concentrations and Standard Deviations (SD) during storage (n = 6).

Folic acid concentration in µg/100 ml (M/SD)		
Initial		186.0/33.6
6 Months	Dark	118.5/19.2
	Light	112.5/13.2
12 Months	Dark	98.9/23.6
	Light	92.4/15.3

months of storage all juices had reached or exceeded their expiration date and we found the following deviations: +4% dark, n = 9; -8% with light, n = 6.

4. Discussion and conclusion

Results from our study reveal a non-linear degradation process of FA with a high decrease in concentration during storage over one year (46%). These findings demonstrate the instability of FA in this aqueous acidic matrix.

The degradation process of FA itself has been described before (Akhtar et al., 1999; Day & Gregory, 1983) and its rate depends on several factors such as temperature, light, pH, oxygen and the overall composition of the respective food product. We observed the highest degradation rates in the first month of our study. Our observations concur with the experimental findings of Saxby et al. (Saxby, Smith, Blake, & Coveney, 1983) where the degradation of FA is investigated in a model buffer solution. The faster degrada-tion rate is presumed to be due to reaction with oxygen (dissolved or present in the head-space of the package) which will eventually be exhausted. Then the degradation proceeds at a lower rate.

Concerning the question of the influence of light on the degradation process of FA, juices in light-transmissive packaging showed a higher degradation when stored under the influence of light than the ones stored in the dark (p = 0.0244).

Nevertheless, these juices in light-transmissive packagings showed hardly any differences in degradation among themselves and the data were not sufficient to attribute a somewhat higher degradation to a certain kind of wrapping (glass or PET).

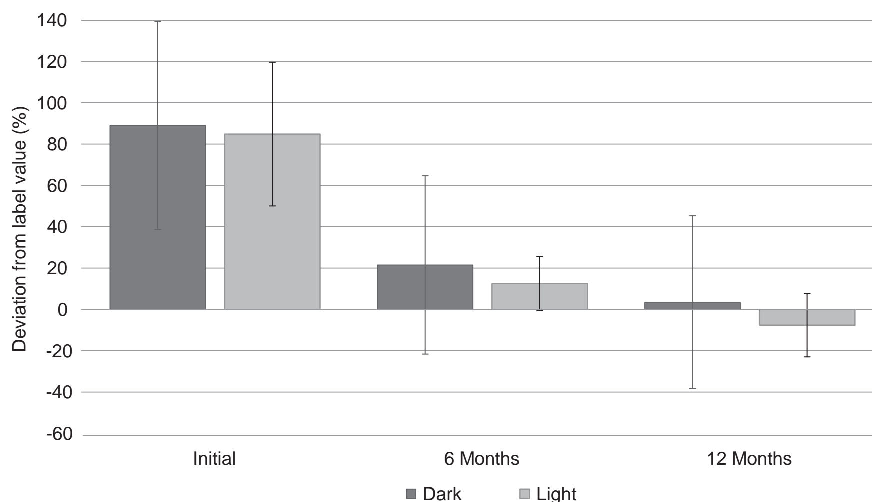

Fig. 3. Deviation from declared label value in fortified juices. Box represents the deviation ($M \pm$ SD) of all juices stored in the dark ($n = 9$) and under the influence of light ($n = 6$), respectively.

Despite this limitation, we were able to reveal that under common storage conditions, which we tried to replicate with our study design, the vitamin intake via fortified fruit juices depends greatly on the storage time before consumption.

We opted for a reliable method which included a quick and simple sample clean-up. HPLC–MS and HPLC–MS/MS methods have been shown to provide specific and reliable data to analyze folates in food samples (Phillips, Ruggio, & Haytowitz, 2011; Vishnumohan, Arcot, & Pickford, 2011): due to increased selectivity HPLC–MS/MS was our method of choice. Nevertheless, due to the complex nature of some foods, a sophisticated sample preparation for the determination of folates is often required. An additional enzymatic treatment is necessary to transform the polyglutamates into the monoglutamate species prior to analyses as most naturally occurring folates are polyglutamates (Rychlik, Englert, Kapfer, & Kirchhoff, 2007).

We focused on the concentration of FA used for fortification and did not analyze naturally occuring folates. As FA is a monoglutamate, we were able to omit the deconjugation step and thus to simplify the sample preparation enabling a high throughput measurement. Our validation data proved this method to be suitable for our needs.

Highest average deviations from the labeled values (up to +89%) were found in the fresh juices from initial analyses. As expected we found much higher concentrations of FA than those declared on the label. These findings are confirmed by similar studies on this subject where different fortified foods (dairy drinks, fruit juices, flour) have been analyzed and in many cases a higher amount of added vitamins than the declared label value has been found (Breithaupt, 2001; Lebiedzinska, Dabrowska, Szefer, & Marszall, 2008; Rodriguez-Comesana, Garcia-Falcon, & Simal-Gandara, 2002). The long term storage design of our study reveals more detailed information in that matter as we analyzed this particular product throughout the entire shelf life.

The average FA concentration found in the fresh juices exceeds the guideline tolerance of +50% (LChG, 2009). Naturally occurring folates in these fruit juices are comparatively low and cannot account for the high concentration as it may be the case with other vitamins (Rodriguez-Comesana et al., 2002). We therefore conclude that a significantly higher amount than recommended by the guiding principles is added in the fortification process. Producers are in a dilemma: on the one hand they have to guaran-tee for the declared vitamin concentration until the products' expiration date and at the same time they deal with the fast degradation of the vitamin in acidic environment. The results of our study give rise to the question whether fruit juices are suitable for the fortification with FA.

The folate or FA intake assessed in nutritional studies is often calculated by using the nutrient labels of the fortified products. In consequence, the high deviations of the declared values make it difficult to determine the correct daily FA intake and lead to an underestimation.

Considering the high doses of FA in some of the freshly-bottled vitamin juices, the tolerable upper intake level (UL) of 1 mg FA/day as given by the European Food Safety Authority (EFSA, 2006) can easily be exceeded by drinking only 600 ml of vitamin juice which is found to be a reasonable amount consumed by some Germans (Hilbig et al., 2009). It is noteworthy that juices can be consumed in large amounts in order to quench one's thirst unlike any other food groups. Our results under the premise of possible risks caused by a high daily FA intake indicate the need for mandatory regula-tions regarding the fortification of beverages with FA.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foodchem.2014.02.156.

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