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Determination of Total Folate in Plant Material by Chemical Conversion into *para*-Aminobenzoic Acid Followed by High Performance Liquid Chromatography Combined with On-Line Postcolumn Derivatization and Fluorescence Detection

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A procedure involving chemical conversion of all forms of folate present in plant material into *para*-aminobenzoic acid (PABA) and a liquid chromatographic-fluorimetric determination with on-line postcolumn derivatization is reported. All folates are cleaved with liberation of PABA by hydrogen peroxide followed by acid hydrolysis using concentrated hydrochloric acid (37%) at 110 °C for 6 h. The reaction yield for individual folates conversion to PABA ranged from 44.4 to 97.3%. PABA could be determined sensitively by on-line postcolumn derivatization with fluorescamine, the detection limit for PABA being 3.02 nM. On the basis of this principle, a method for the determination of total folate in plant material, including a purification step on an affinity column, is presented, which offers a sufficient sensitivity and selectivity for routine analysis of total folate in natural samples. The total folate contents of tomatoes, carrots, white cabbage, and spinach were determined, and the results were quite comparable to the data reported. The recovery of PABA and the comparison of total folate analysis in spinach on different occasions (over 6 months) are also reported. The method is reliable, universal for all folates, including polyglutamate and monoglutamate forms, and eliminates the need for a deconjugation step and multiple conversion reactions.

KEYWORDS: Folates; PABA; high performance liquid chromatography; postcolumn reaction; fluorescence; fluorescamine

INTRODUCTION

Folate is a water-soluble vitamin (B_9) that plays a key role in the methylation cycle and in DNA biosynthesis. Folate deficiency has been implicated in hyperhomocysteinemia, which results in an increased risk of cardiovascular disease and dementia and in neural tube defects (*1*−*3*).

Reliable data of the folate level in different specimens, such as human blood and plant materials, is of much importance for clinical and nutritional issues. However, the folate content in natural samples is often very low, consequently requiring a sensitive assay. The identification of naturally occurring folates is complicated by the large number of possible derivatives arising from combinations of oxidation state, one carbon moiety, and polyglutamate chain length, as well as by the presence of breakdown products that arise during extraction and storage (*4*). HPLC combined with sensitive detection enabled qualitative and quantitative analysis of natural folates (*5*−*16*). Among these methods, HPLC-UV seems to be universal for all folates. However, it is not sensitive enough to detect all natural folates, because the levels of some forms remain far below the detection limit (*10*). Some reduced forms of folates have fluorescence properties, such as 5-methyltetrahydrofolate and tetrahydrofolate (λ_{ex}, 290 nm; λ_{em}, 356 nm) and 10-formylfolic acid (λ_{ex}, 360 nm; λ_{em}, 460 nm). However, folic acid itself and other folates with different glutamic acid chain lengths are not fluorescent (*10*−*13*). Other assays based on electrochemical (*14*−*16*) or chemiluminescence detection (*17*) were also proposed, but none of these methods is universal to detect all forms of naturally occurring folates. HPLC-MS is a sensitive and universal assay for all folates (*18*−*26*), and clearly offers possibilities for the future. However, this technique is not always accessible in research laboratories.

On the basis of these considerations, the ability to quickly and reliably determine the total amount of folates in biological samples is still of great importance. The microbiological assay

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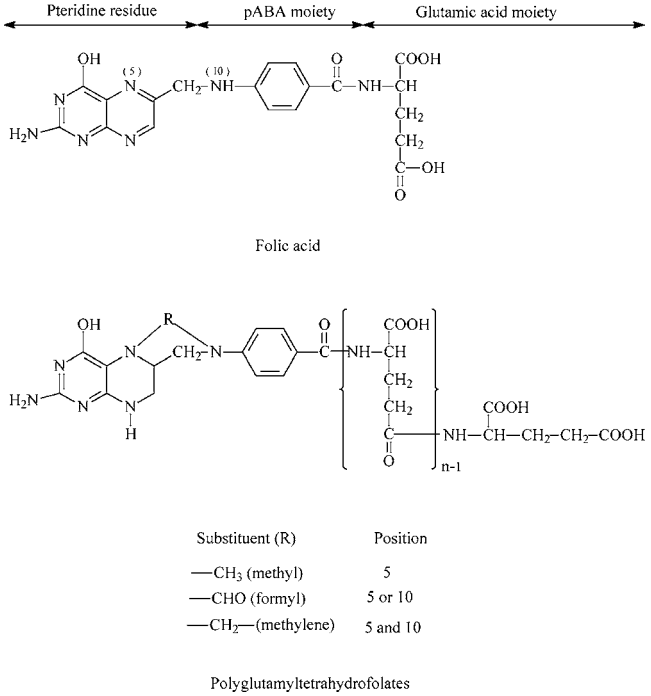

Figure 1. Chemical structure of folates.

is by far the most commonly used method and has been referred to as the "gold standard" for this purpose (*1*). However, polyglutamates should be deconjugated to monoglutamates, because microorganisms respond mainly to monoglutamates. Additionally, matrix components could stimulate or inhibit bacterial growth, occasionally resulting in less reliable data (*27*).

Ndaw et al. (*28*) reported a method to convert all folates into 5-methyltetrahydrofolate through 5 reaction steps for 4 groups of folates. They used HPLC with fluorescence detection to assess the total folate content by the determination of 5-methyltetrahydrofolate (having the highest fluorescence yield). However, special care should be taken in this procedure, because 5-methyltetrahydrofolate is an unstable and easily oxidized compound. In addition, the long and complex procedure requires much experience.

The radioprotein-binding assay (RPBA) based on binding affinity of a folate binding protein (FBP) for the folates has been modified recently to analyze total folate in various food samples (*29*−*31*).

We present a simpler, specific, and reliable alternative method for determining the total folate content in biological samples based on a method reported and applied only to blood samples (*32*−*35*). Folates consist of a pteridine residue, PABA, and a different number of glutamic acid residues (**Figure 1**). As all folates have the same part, PABA, in their structures, it is possible to cleave them and to analyze total folate by determining PABA. On the basis of the method reported (*32*−*35*), folates in blood can be hydrolyzed into PABA in concentrated HCl at 110 °C. However, contrary to the yields obtained in blood sample analyses (*32*−*35*), the present work showed that the reaction yield of this hydrolysis was very low for some folate standards, especially for 5-methyltetrahydrofolate and 5-methyldihydrofolate, which shows that the virtue of their method is limited to the blood sample. This is a major drawback, because it has been demonstrated that the only significant form found in human circulation is 5-methyltetrahydrofolate, as a monoglutamate (*2*). Also, in plants, 5-methyltetrahydrofolate was found to be the predominant form (*36*). To solve this problem, we developed a chemical method to increase hydrolysis reaction yield and used HPLC and postcolumn derivatization to determine the hydrolysis product (i.e., PABA). With this method, we determined the total folate content in different vegetables.

Table 1. Results and Conditions for the Determination of Purity of Folate Standards by UV

standard	pH[a]	ϵ^b (10^4 L mol^{-1} cm^{-1})	λ_{max} (nm)	purity (%)
5-MTHF	7.0	3.17	290	61.8
THF	7.0	2.91	297	64.0
5-CHO-THF	7.0	3.72	285	62.8
10-CHO–PteGlu	7.0	2.09	269	89.4
5,10-methyleneTHF	7.2	3.20	294	91.1
DHF	7.0	2.84	282	74.9
PteGlu	7.0	2.76	282	76.3
$PteGlu_4$	7.0	2.76	282	62.0
5-MDHF	7.0	3.12	290	82.2

[a] The pH of the standard solution was adjusted with 0.01 M phosphate buffer. [b] Molar absorptivities obtained from ref 37.

MATERIALS AND METHODS

Standards and Chemicals. PABA (Sigma, Bornem, Belgium) was a kind gift from Prof. Van Den Bossche (Ghent University, Fac. Pharm. Sci.). PABA stock solution (1.0 g/L) was prepared in methanol, and each standard solution of PABA was obtained by dilution of this stock solution using eluent A (98% 0.01 M acetate buffer adjusted to pH 4.75 with acetic acid, and 2% acetonitrile).

The following folates were purchased from Schirck's Laboratories (Jona, Switzerland): 5,10 -methylenetetrahydrofolate (5,10-methyleneTHF), 10-formylfolic acid (10-CHO−PteGlu), 5-methyl-5,6-dihydrofolate (5-MDHF), 5-formyltetrahydrofolate (5-CHO-THF), and folic acid polyglutamates ($PteGlu_n$, $n = 2-8$). 5-Methyltetrahydrofolate (5-MTHF), tetrahydrofolic acid (THF), and dihydrofolate (DHF) were from Sigma (Bornem, Belgium). Folic acid (PteGlu) was from Janssen Chimica (Geel, Belgium).

Hydrogen peroxide (37%), $KMnO_4$, acetonitrile for HPLC, and FBP were obtained from Sigma (Bornem, Belgium). Agarose for gel affinity chromatography (Affi-gel 10) was from Bio-Rad Labs (Eke-Nazareth, Belgium) and kept at −18 °C. Fluorescamine was purchased from Fluka (Bornem, Belgium) and kept at −5 °C. Water used throughout this study was HPLC grade. All other chemicals used were of the highest purity available.

Purity Verification of Folates and Preparation of Stock Solutions. Samples (1.0×10^{-3} g/L) of each folate standard solution were prepared directly in 0.01 M phosphate buffer (pH = 7.0, except pH = 7.2 in the case of 5,10-methylene THF). The purity of each standard was verified using UV spectrophotometry with 0.01 M phosphate buffer as blank, and the purities were calculated using the respective molar absorption coefficients (*37*) (**Table 1**). For UV measurements, ascorbic acid should be avoided, because it interferes with the absorbance of the folates. In addition, the whole process should be performed rapidly, due to the extreme instability of folates in aqueous medium without any antioxidant (*11*).

Stock solutions of each folate contained 5.0×10^{-2} g/L and were prepared in a 1.0% aqueous ascorbic acid (Sigma, Bornem, Belgium) solution. The reaction yield of each folate was determined and calculated using this freshly prepared stock solution.

Sample Preparation. Different fresh vegetables were purchased from local supermarkets. White cabbage and spinach were mainly sampled from their stems and leaves. Carrots and tomatoes were sampled directly, without leaves. During the sample preparation, all operations before hydrolysis were carried out under subdued yellow light.

Folate Extraction. The folate extraction procedure was a modification of the method of Vahteristo and co-workers (*11*). A sample (1−2 g) was weighed and cut into pieces (0.5−1 cm), 30 mL of 100 mM phosphate buffer (pH = 7.0) containing 1.0% ascorbic acid was added, then the pH was adjusted to 6.0 with phosphoric acid. The sample was

homogenized using an Ultra-Turrax homogenizer for 1 min under a nitrogen flow. During this process, 1.0 mL of 2-octanol was added to reduce foam formation. The homogenate was then placed in a water bath at 100 °C for 10 min, and the tube was shaken twice during this extraction. The extracts were rapidly cooled in ice and filtered through a 0.2-μm filter under vacuum. The residue was redissolved in 5−10 mL of extraction buffer; the solution was collected and filled to 50 mL with extraction buffer in a volumetric flask. The extract was stored at −18 °C until analysis (not longer than two weeks after extraction).

Preparation of the Affinity Column. This procedure was according to Konings, with minor modifications (*13*). In brief, 1.0 mL of FBP (1.0 mg/mL prepared with 120 mM phosphate buffer (pH = 7.4, including 30 mM sodium chloride) with 1.5 mL of cold $NaHCO_3$ (0.1 M, pH = 6.5) was taken in a 10-mL capped bottle. Three bed volumes of a cold sodium acetate solution (0.01 M, pH = 4.5) were used to wash 2.0 mL of Affi-Gel, and vacuum was applied, without drying the gel. The moist gel cake was transferred to the cold FBP solution within 20 min. The solution was gently agitated for 4 h at 4 °C, then 0.2 mL of an aqueous ethanolamine solution (1.0 M, pH = 8.0) was added, and it was continuously agitated at 4 °C for 1 h. The gel was distributed over a glass column, and washed two times with 5 mL of phosphate buffer (0.1 M, pH = 7.0). When not in use, the column was stored at 4 °C in phosphate buffer (0.1 M, pH = 7.0) containing 0.2% (W/V) sodium azide.

Purification of Samples Using Affinity Chromatography. The FBP column was equilibrated by rinsing with 1.0 mL of phosphate buffer (0.1 M, pH = 7.0). Then, 3.0 mL of the sample solution was loaded onto the column, and 5.0 mL of 0.025 M phosphate buffer (pH = 7.0, containing 1.0 M NaCl) was taken to rinse the column, followed by 5.0 mL of 0.025 M phosphate buffer (pH = 7.0). The folates were eluted with 4.6 mL elution solution (0.02 M trifluoroacetic acid−0.02 M dithioerythritol). The elution solution was transferred to a 5.0-mL volumetric flask and was filled to the mark with elution solution. The capacity of the folate affinity column was 1.0 μg (based on 5-MTHF binding). To get a satisfactory binding with all folates (recoveries above 90%), the folate load should not exceed 25% of the column capacity, according to Kariluoto (*38*).

Chemical Conversion of Folates into PABA by Oxidation and Acid Hydrolysis. A 500-μL aliquot of the above elution solution was taken for hydrolysis, 40 μL of 2.0 M H_2O_2 was added, and the sample was left at 100 °C for 10 min. After cooling the solution to room temperature, 100 μL of 7.2 mM $KMnO_4$ was added into the above solution. The solution was mixed well by shaking for 1 min, then it was dried completely using N_2 at room temperature. A 1 mL sample of 37% HCl was added, and the mixture was transferred in a capped 10-mL tube for hydrolysis for 6 h at 110 °C. The reaction mixture was cooled to room temperature and dried completely at 60 °C under N_2. Finally, 200 μL of eluent A (98% 0.01 M acetate buffer adjusted to pH 4.75 with acetic acid, and 2% acetonitrile) was added for HPLC analysis.

Chromatographic Determination. The HPLC system consisted of an L-7100 pump, an L-7200 autosampler, an L-7485 fluorescence detector, a D-7000 interface connected with a computer, a postcolumn reagent delivery pump (L-6200 intelligent pump), and a column oven (L-7360). The entire system was from Merck (Leuven, Belgium).

A Purospher Star RP-18 endcapped column (150 × 4.6 mm i.d..; octadecylsilyl 5-μm particle size; Merck, Darmstadt, Germany), and a guard column RP 18 (4 mm × 4 mm i.d.; octadecylsilyl 5 μm particle size; Merck, Darmstadt, Germany) were used for all analyses. The postcolumn derivatization solution was delivered to a reaction coil (Teflon tube, 5 m × 0.25 mm i.d. × 1.59 mm o.d.) through a T-shaped connector, the reaction coil was put in a column oven at 42 °C.

Separation of PABA from the Hydrolysis Products. The mobile phase used was a gradient of eluent A (98% 0.01 M acetate buffer adjusted to pH 4.75 with acetic acid and 2% acetonitrile) and eluent B (68% 0.01 M acetate buffer adjusted to pH 4.75 with acetic acid and 32% acetonitrile). The starting eluent was 98% A mix with 2% B, the proportion of B was increased linearly to 17% in 15 min, then to 95% in 15 min. The mobile phase was immediately adjusted to its initial composition and held for 10 min to reequilibrate the column. The flow rate of the mobile phase was 0.7 mL/min, and the injection volume was 50 μL. The column was kept at 42 °C with a column oven. Under these conditions, the retention time of PABA was 12.8 min.

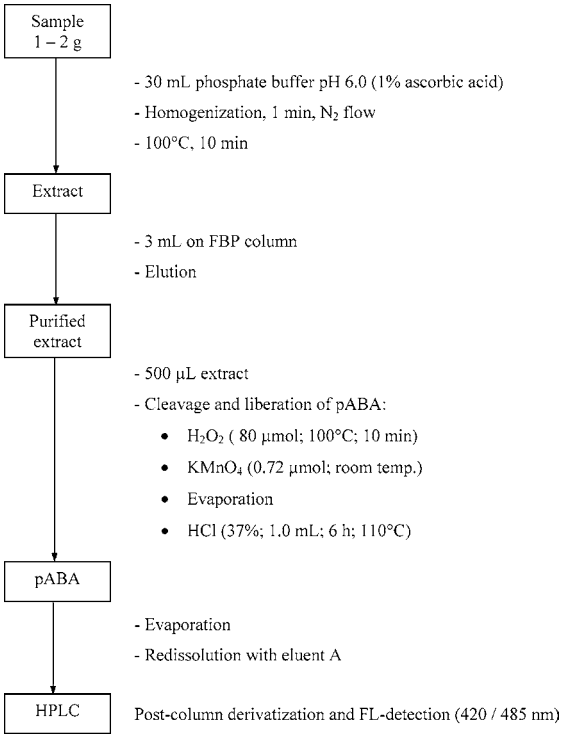

Figure 2. Schematic overview of the procedure.

On-Line Postcolumn Derivatization for Fluorescence Determination. For the determination of PABA with fluorescence detection, an on-line postcolumn derivatization process was carried out. PABA is derivatized to a highly fluorescent compound by reaction with 8.98×10^{-4} M fluorescamine in 0.02 M NaH_2PO_4 (adjusted to pH 3 with phosphoric acid) (62.5 mg fluorescamine was dissolved in 25% (V/V) acetonitrile, and 0.2% (V/V) 2-mercaptoethanol was added, finally the solution was diluted to 250 mL in a flask with phosphate buffer pH = 3.0). During the whole experiment, the solution of fluorescamine was kept at 5 °C in a refrigerator. The flow rate of the postcolumn derivatization reagent was 0.2 mL/min. The fluorescence detection was performed at an excitation wavelength of 420 nm and at an emission wavelength of 485 nm.

The whole procedure is summarized in **Figure 2**.

RESULTS AND DISCUSSION

Conversion of Folate Standards into PABA. Dueker et al. and Lin et al. (*33*−*35*) described an acid hydrolysis-HPLC-GCMS method to determine folates in whole blood by monitoring a derivative of PABA. Their method compared quite well with the microbiological method for the determination of total folate in whole blood. Likewise, we developed a method based on the idea that the total folate content in a sample can be determined through the determination of PABA after hydrolysis of the folate. Conversion of folate standards into PABA was carried out according to these previous reports (*33*−*35*). The results showed that all folates could be hydrolyzed into PABA. However, the reaction yield for some folates was really low, especially for 5-MTHF and 5-MDHF with yields below 15% (**Table 2**, method a). However, 5-MTHF is usually the most predominant folate derivative (*36*), implying that a low reaction yield for 5-MTHF will hamper its accurate determination with this method. According to the reports of Dueker et al. and Lin et al. (*33*−*35*), the hydrolysis of exogenously added folic acid in blood should be almost complete (mean recovery from a blood sample was 96.7%). In the absence of blood, however,

Table 2. Prevalence in Plant Materials and Reaction Yield for Representative Folates in Different Hydrolysis Methods

folate	prevalence in plant material (%)[a]	reaction yield (%) (method a)[b]	reaction yield (%) (method b)[c]
5-MTHF	83.6∼94.1	12.2	97.3
THF	3.8∼16.9	49.8	69.7
5-CHO-THF	∼5.2	18.5	79.9
10-CHO–PteGlu	∼0.85	65.2	44.4
5,10-methylene-THF	not reported	61.4	90.0
DHF	not reported	78.1	90.5
PteGlu	not reported	76.4	88.6
$PteGlu_4$	not reported	96.1	87.4
5-MDHF	not reported	5.0	66.7

[a] Data from ref 36. [b] Hydrolysis with 1.0 mL of 37% HCl at 110 °C for 6 h; fluorescence detection at 485 nm with excitation at 420 nm (*33*–*35*), applied to folate standards without the presence of blood. [c] Hydrolysis with 1.0 mL of 37% HCl at 110°C for 6 h before hydrolysis folates reacted with 40 μL 2.0 M H_2O_2 at 100°C for 10 min, then 100 μL 7.2 mM $KMnO_4$ was added to remove any excess of H_2O_2. Fluorescence detection at 485 nm with excitation at 420 nm.

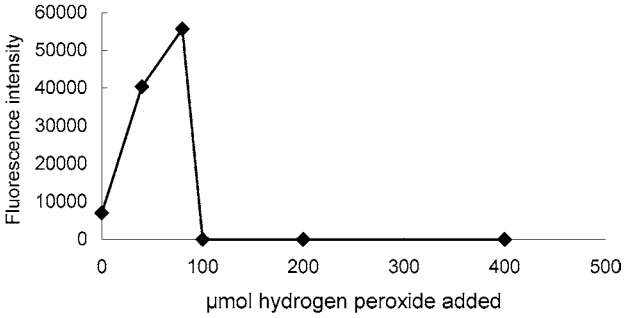

Figure 3. The effect of the amount of H_2O_2 on the hydrolysis. Stock solution (1.26×10^{-3} g/L) of 5-MTHF was used. The reaction with H_2O_2 was carried out at 100 °C for 10 min, and the following hydrolysis was at 110 °C using 37% HCl for 6 h.

in our hands, only approximately 76% of this folate form, and even much less for other folates could be hydrolyzed into PABA.

Blood is a special matrix for acid hydrolysis of folates. The iron (III) in blood was suspected to be a catalyst for the hydrolysis reaction. We evaluated iron (III), Zn dust + HCl, and H_2O_2 as enhancers for this reaction. Zn dust had a negative effect, which was opposite for 5-MTHF compared to folic acid as reported by Shane (*4*). Hydrogen peroxide enhanced the hydrolysis more than iron (III), indicating that the mechanism of the effect of blood on the hydrolysis was complex. Hydrogen peroxide was chosen for further experiments in the present work. However, as demonstrated in **Figure 3**, an excess of hydrogen peroxide will destroy PABA completely during the hydrolysis reaction. The destruction of PABA by hydrogen peroxide was also demonstrated using standard solutions of PABA under the same conditions.

When dealing with different samples containing varying amounts of folate, the precise amount of hydrogen peroxide is hard to control, and any excess of hydrogen peroxide will destroy the reaction product of the folate hydrolysis (i.e., PABA). However, it was demonstrated that the reaction of the folates with hydrogen peroxide happened before acid hydrolysis rather than during the hydrolysis (data not shown), implying that excess of hydrogen peroxide will not react with PABA before acid hydrolysis. Thus, to improve the reliability of the procedure, it is preferable to eliminate the excess of hydrogen peroxide before acid hydrolysis. As shown in the insert of **Figure 4**, $KMnO_4$ can eliminate this excess of hydrogen peroxide preventing further destruction of PABA. In addition, a small excess of $KMnO_4$ has no negative effect on the final yield, but even slightly improves it (**Figure 4**). The same figure also demonstrates that very high amounts of hydrogen peroxide and $KMnO_4$ will decrease the amount of PABA formed. The optimum amounts of hydrogen peroxide and $KMnO_4$ chosen in this work are 80 and 0.72 μmol, respectively. Under these conditions, PABA production from folates ranges from 44.4 to 97.3% (method b in **Table 2**). Especially for 5-MTHF and 5-MDHF, reaction yields were much improved compared to those without the addition of hydrogen peroxide (method a in **Table 2**). The chromatograms of the hydrolysis product of some of the folates under these conditions are shown in **Figure 5**.

PABA Derivatization and Its Stability. Fluorescamine is a fluorogenic reagent specific for primary aliphatic and aromatic amines, producing fluorophors with a high fluorescence yield. In the present work, PABA as an aromatic amine showed high fluorescence after being derivatized with fluorescamine similar to other primary amines (*39*). To achieve the maximum signal-to-noise ratio, 0.2 mL/min of 8.98×10^{-4} M fluorescamine in 0.02 M phosphate buffer (pH = 3.0) was adopted in the present work.

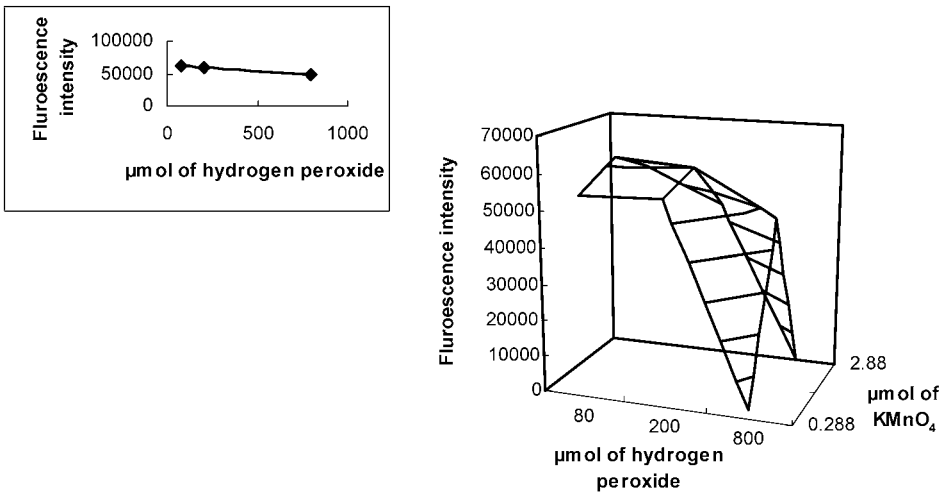

Figure 4. The effect of varying amounts of hydrogen peroxide and $KMnO_4$ on the hydrolysis yield. The insert is the effect of hydrogen peroxide on the hydrolysis yield, with 100 μL of 7.2 mM $KMnO_4$ added. The reaction of 5-MTHF with hydrogen peroxide at 100 °C for 10 min was followed by adding different amounts of $KMnO_4$. The other conditions are the same as those in **Figure 3**.

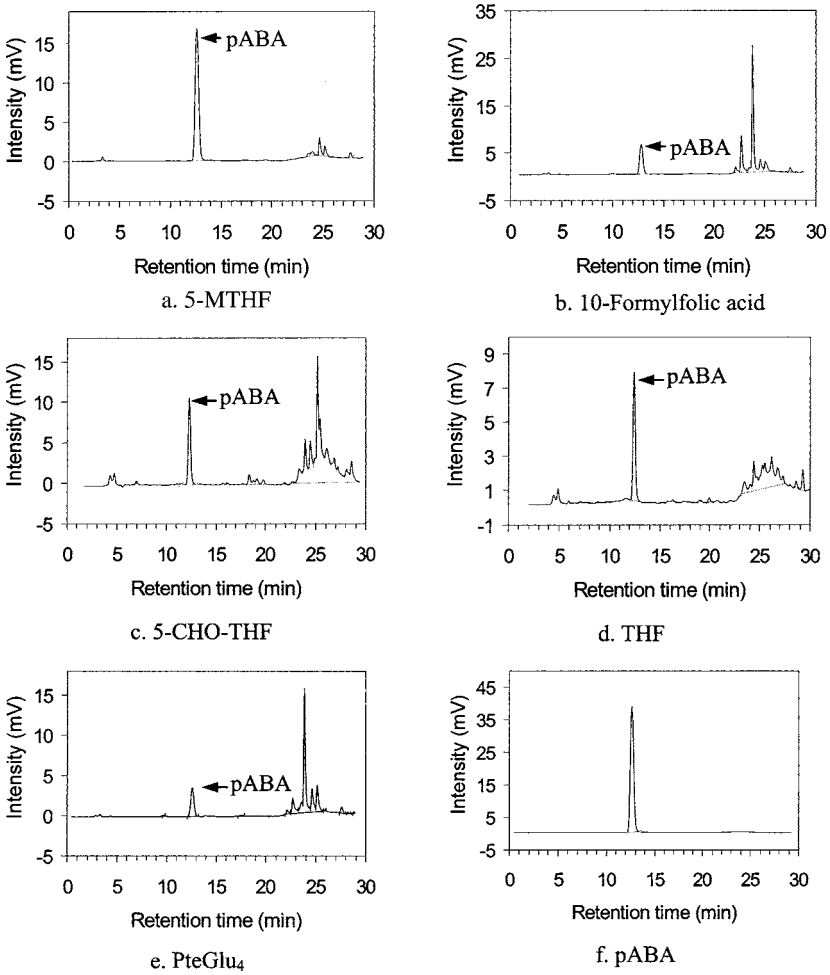

Figure 5. Chromatograms of the hydrolysis product of some folate standards and PABA. 2.6×10^{-4} g /L 5-MTHF (a), 1.5×10^{-4} g/L10-formylfolic acid (b), 2.5×10^{-4} g/L 5-CHO-THF (c), 2.9×10^{-4} g/L THF (d), 1.5×10^{-4} g/L pteroyltetra-γ-glutamic acid (e), and 1.04×10^{-4} g/L PABA standard (f), using a gradient of eluent A (98% 0.01 M acetate buffer adjusted pH to 4.75 with acetic acid, and 2% acetonitrile) and eluent B (68% 0.01 M acetate buffer adjusted pH to 4.75 with acetic acid, and 32% acetonitrile). The column was kept at 42 °C. The postcolumn derivatization reagent was 8.98×10^{-4} M fluorescamine in 0.02 M NaH_2PO_4–H_3PO_4 buffer (pH = 3), and fluorescence detection was at 485 nm with excitation at 420 nm.

The stability of PABA during the hydrolysis reaction was also investigated. The difference between the results obtained from the initial determination and a second one after 12 h was only 1.93% (data not shown). After 12 h, the fluorescence intensity decreased because of partial degradation of the postcolumn derivatization reagent. Therefore, this reagent was prepared freshly on a daily basis and the solution of fluorescamine was stabilized with 0.2% (V/V) 2-mercaptoethanol as an anti-oxidant and kept at 5 °C during the whole experiment.

The Necessity of Purification on an Affinity Column before Sample Analysis. The purification of the samples before HPLC analysis is necessary because of interferences and possible naturally occurring PABA in sample. There are mainly two purification methods applied to folate analysis, the first one being strong anion-exchange solid-phase extraction as reported by Vahteristo et al. (*11*), the second one being immuno-affinity chromatography based on folate binding protein, as developed by Selhub et al. (*40*) and adapted by Gregory et al. and others (*41*−*42*). The latter method is clearly more specific than the former one and was preferred here. Our experiments showed that ascorbic acid present in the extraction buffer had a negative effect on the hydrolysis yield and had to be removed before this hydrolysis. The immuno-affinity column not only could remove ascorbic acid from the extract solution, it could also remove the naturally occurring PABA in the sample. In this way, contribution of initially present PABA was eliminated.

Characteristics of the Proposed Method. A calibration curve using different amounts of PABA was set up and the peak area was used for quantification. The fluorescence intensity was linear to the concentration of PABA ranging from 7.55 to 3025 nM with slope of 0.14 and intercept of −0.98. The RSD was 1.43% by determining a sample containing 7.55 nM PABA in triplicate. The detection limit and the correlation coefficient r^2 are 3.02 nM (S/N = 3) and 0.9996, respectively.

Because the production yield of each folate has been determined using the whole experimental process, and because purification by FBP was performed according to the literature (13, 40−42), PABA was used for recovery experiments without the affinity chromatographic step. Five determinations with PABA added (final concentration of PABA: 1.43×10^{-8} M) yielded an average recovery and RSD of 101 and 5.91%, respectively.

Determinations of the Total Folate Content in Some Vegetables. Because all folates can be hydrolyzed into PABA, the determination of PABA was used to assess the total amount of folates in biological samples. Some vegetables such as tomatoes, carrots, white cabbage, and spinach were chosen to test this method. **Figure 6** shows representative chromatograms of the analysis of tomatoes and carrots, and **Table 3** presents the mean total folate values of the samples analyzed, which was compared with the HPLC data (*27*, *28*, *36*) and the

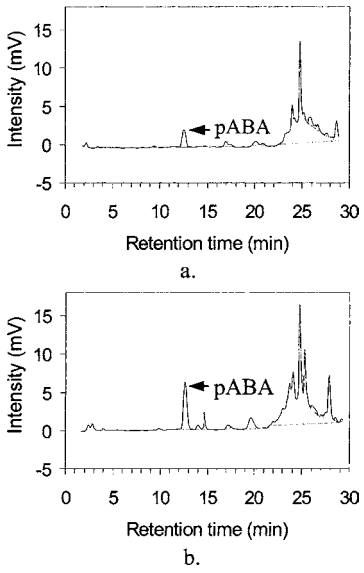

Figure 6. Representative chromatograms for the analysis of (a) tomatoes, and (b) carrots. Chromatographic conditions: see legend to **Figure 5**.

Table 3. Total Folate Content of Some Vegetables

sample	total folate content (μg/100 g)[a]	RSD (n=3)[b] (%)	literature data (μg/100 g)[d]	USDA data (μg/100 g)[e]
tomatoes	4.1	4.85	8 (*27*)–11 (*36*)	15
carrots	13.8	7.53	13 (*27*)–16 (*36*)	19.4
white cabbage	32.6	13.5	24 (*27*)[c]–52 (*36*)	42.8
spinach	142.4	9.64 (n=5)	114 [28]–150 (*1*)	190

[a] Calculated based on molecular weight of folic acid (441.4). Mean of three determinations. [b] For each analysis, a new original sample was processed. [c] Information obtained for red cabbage. [d] Numbers in parentheses indicate reference numbers. [e] Ref 43.

Table 4. Evaluation of the Method by the Determination of the Total Folate Content in Spinach at Different Occasions

samplings[a]	n	total folate content (μg/100 g)[b]	intra assay RSD (%)	inter assay RSD (%)
first determination	5	142.4	9.64	5.84
3 months later	3	139.4	8.56	
5 months later	3	137.6	11.4	
6 months later	3	124.4	4.9	

[a] Fresh spinach was purchased from the same local supermarket (the package is sealed with a plastic bag) every time before analysis. [b] Mean of total folate content calculated based on molecular weight of folic acid (441.4).

microbiological data from the U.S. Department of Agriculture (USDA) (*43*).

As mentioned, the hydrolysis reaction yield for individual folates is not identical (**Table 2**). However, the high prevalence of 5-MTHF in plant material minimizes the contribution of the other folate analogues.

For further evaluation of the method, the total folate content in fresh spinach (for avoiding losses of folate after long time of storage, the same fresh product from the same supermarket was purchased for each determination) was also evaluated on different occasions over a period of 6 months. The mean values, number of determinations, and RSD values are shown in **Table 4**, and indicate an acceptable reproducibility.

Our modifications make the elegant procedures for blood as described by Dueker et al. and Lin et al. (*33*−*35*), applicable to plant material and make this method another option for total folate determination overcoming the effect of sample matrix. The final product, PABA, is much more stable than the individual folate derivatives, allowing the use of an autosampler without cooling system (*11*). In addition, the incorporation of the immuno-affinity purification step enhances sensitivity (*42*) and selectivity for the folate derivatives, thus making the described method now applicable to natural samples.

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