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Determination of folates in foods by high-performance liquid chromatography with fluorescence detection after precolumn conversion to 5-methyltetrahydrofolates

S. Ndaw[a], M. Bergaentzlé[a], D. Aoudé-Werner[b], S. Lahély[a,1], C. Hasselmann[a,*]

[a]*Laboratoire de Chimie Analytique et Sciences de l'Aliment (UMR 7512), Faculté de Pharmacie, 74 Route du Rhin, 67400 Illkirch, France*

[b]*Aérial, 19 Rue de Saint Junien, BP 23, 67305 Schiltigheim Cedex, France*

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Abstract

A liquid chromatographic–fluorimetric determination of folates in foodstuffs including their extraction, without or with deconjugation, chemical conversion to 5-CH_3-H_4PteGlu$_n$ and purification of the extract by affinity chromatography is reported. The conversion enables the analysis of total folates and also of the contents of the different mono- and polyglutamate forms of the folates. The method has a satisfactory day-to-day repeatability (never more than 10%) and a very low detection limit (0.02 pmol per injection). Depending on the folate studied, the recovery rates varied from 78% (10-CHO-PteGlu) to 98% (5-CHO-H_4PteGlu). Furthermore it has been possible to show that the deconjugation of the folates by rat plasma conjugase was incomplete in foodstuffs whereas chicken pancreas conjugase effectively converted the different folate polyglutamates into folate diglutamates. It could not be demonstrated that prior hydrolysis with a protease and amylase was useful for the analysis of the different foodstuffs studied (yeast, spinach, beef liver, beef fillet and peas) when deconjugation was performed with the chicken pancreas conjugase.

Keywords: Folates; 5-Methyltetrahydrofolates

1. Introduction

The most widely used analytical procedure for the determination of the total folate content of foods is still the microbiological assay using *Lactobacillus casei* as test organism, where a growth response of the organism to the mixture of folates present is measured turbidimetrically [1,2]. However, even with the development of semi-automated procedures including the microtitration format [3,4], the microbiological approach is both time-consuming and demanding in execution. Additionally, the response of the organism to the different folate forms is not always identical.

Subsequent to studies initiated by Gregory and

*Corresponding author. Tel.: +33-3-9024-4160; fax: +33-3-9024-4100.

E-mail address: claude.hasselmann@pharma.u-strasbg.fr (C. Hasselmann).

[1]Present address: Garden State Nutritionals, 100 Lehigh Drive, Fairfield, NJ 07004, USA.

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co-workers [5–7], the analysis of folates in foodstuffs by high-performance liquid chromatography (HPLC) techniques has been very widely developed and has recently been the subject of many publications [8–14]. The analytical protocols suggested usually include a trienzyme (conjugase, protease, α-amylase) treatment of the sample, a purification of the extract by affinity chromatography and a separation of the folate monoglutamates by reversed-phase HPLC. However, the multiplicity and diversity of natural folates, their existence at low concentrations in foodstuffs, their possible destruction by light, heat and/or oxygen and the low fluorescence quantum yield of some folates present obstacles to the liquid chromatographic separation and fluorescence quantification of these compounds. In fact, these chromatographic procedures are generally used to obtain some information concerning the folate composition of foodstuffs for a good evaluation of bioavailability studies.

Hence, in order to make possible a chromatographic analysis of folates in foodstuffs, a simple, rapid and quantitative precolumn chemical conversion of all of the folates present into 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu) is proposed, the latter being then easily isolated by liquid chromatography and analyzed by fluorescence. The choice of 5-CH_3-H_4PteGlu suggested itself for several reasons. This chemical compound, very stable in acidic medium, is in fact the folate possessing the highest fluorescence quantum yield [15], hence the one which it is possible to analyze with the highest sensitivity. Moreover, several reaction schemes seem to enable it to be produced from folic acid (PteGlu) [16],5-formyltetrahydrofolate(5-CHO-H_4PteGlu)[17] and 10-formylfolate (10-CHO-PteGlu) [18].

Such a precolumn conversion performed on food samples not subjected to deconjugation ought moreover to make possible a separation of the different 5-CH_3-H_4PteGlu polyglutamates (5-CH_3-H_4PteGlu$_n$) formed from the folates initially present in this sample. It ought then to be easy to assess the real efficiency of the various conjugases (rat plasma, hog kidney, chicken pancreas) usually recommended and hence to suggest the most satisfactory enzymatic treatment possible for the extraction of the folates from foodstuffs.

2. Experimental

2.1. Standards

The folate standards were obtained from Schirck's Labs. (Jona, Switzerland) [10-formylfolate (10-CHO-PteGlu); 5,10-methenyltetrahydrofolate (5,10-CH^+-H_4PteGlu); 5,10-methylenetetrahydrofolate (5,10-CH_2-H_4PteGlu); folic acid polyglutamates (PteGlu$_n$, $n=2$ to 8)] and Sigma (Saint-Quentin Fallavier, France) [folic acid (PteGlu); tetrahydrofolate (H_4-PteGlu); 5-methyltetrahydrofolate (5-CH_3-H_4PteGlu); 5-formyltetrahydrofolate (5-CHO-H_4PteGlu)]. 10-Formyltetrahydrofolate (10-CHO-H_4PteGlu) was prepared from 5,10-CH^+-H_4PteGlu according to the method proposed by Temple Jr. and Montgomery [19].

2.2. Enzymes and chemicals

The following conjugases were used: chicken pancreas conjugase (Difco Labs., Detroit, MI, USA, catalogue No. 0459-12-2), rat plasma conjugase from a local university animal laboratory, prepared according to the protocol of Konings [12] and hog kidney conjugase, prepared from fresh pig's kidneys as previously described by Pedersen [20]. Each conjugase preparation was assayed for its activity using PteGlu$_n$ ($n=2$, 3, 5, 7, approximately 35 to 80 nmol) as substrates [rat plasma: 500 μl in 30 ml of 100 m*M* phosphate buffer (pH 7) containing 1% L-ascorbic acid, 2 h at 37°C; hog kidney: 500 μl in 30 ml of 100 m*M* phosphate buffer (pH 4.5) containing 1% L-ascorbic acid, 2 h at 37°C; chicken pancreas: 5 mg in 30 ml of 100 m*M* phosphate buffer (pH 7) containing 1% L-ascorbic acid, 1 h at 37°C]. Folic acid mono- or diglutamate formed was analyzed by means of UV absorption after isolation by HPLC [21].

Other enzymes used were pronase (EC 3.4.24.31, Sigma, catalogue No. P5147) and α-amylase from *Aspergillus oryzae* (EC 3.2.1.1, Sigma, catalogue No. A0273).

Sodium borohydride, formaldehyde (37%) and folate binding proteins were obtained from Sigma. Acetonitrile (for HPLC) was obtained from Carlo

Erba (Milan, Italy). Agarose for gel affinity chromatography (Affi-gel 10) was obtained from Bio-Rad Labs. (Marnes-la-Coquette, France). All other chemicals used were of the highest purity available.

2.3. Sample preparation

The foods studied (frozen peas, frozen spinach, powdered milk, yeast, wheat flour, liquid fruit juice, egg yolk, apples, fresh beef liver and fillet) were purchased at local sources. The certified reference materials [pig's liver (CRM 487) and milk powder (CRM 421)] were obtained from the Institute for Reference Materials and Measurements (IRMM, Geel, Belgium). Only fruit juice was supplemented with folic acid. With the exception of dry products (powdered milk, yeast, wheat flour), all foodstuffs were stored frozen until they were analysed. The solid samples were finely ground. The sample amounts [1 g of yeast or beef liver, 2.5 g of pig's liver (CRM 487), 10 g of apple or beef fillet, or 5 g of other foodstuffs] took into consideration the estimated folate content of the food studied. During the sample preparation, all the operations should be carried out under subdued light.

2.3.1. Folate extraction

2.3.1.1. Protocol (a). Extraction without enzymatic treatment

The sample was weighed into a 100-ml conical flask. A 30-ml volume of 100 m*M* phosphate buffer (pH 7) containing 1% L-ascorbic acid was added and the mixture was placed in a water bath at 100°C for 10 min (this step was omitted during the analysis of wheat flour samples to avoid the formation of a doughy mixture). After being allowed to cool, it was diluted to 50 ml with 100 m*M* phosphate buffer (pH 7) containing 1% L-ascorbic acid (during the analysis of powdered milk samples, the pH was first lowered to 4.5 by addition of 5 *M* hydrochloric acid in order to precipitate proteins) and centrifuged at 5000 rpm for 10 min. A 5–10 ml volume of the supernatant was placed in a 150-ml conical flask (the pH was then adjusted to 7 with 5 *M* sodium hydroxide for the powdered milk samples).

2.3.1.2. Protocol (b). Extraction with deconjugation treatment

A 5–10 ml volume of the supernatant obtained in protocol (a) was placed in a 150-ml conical flask. A 1-ml volume of a 5 mg ml^{-1} solution of chicken pancreas conjugase (or 500 μl of rat plasma conjugase) was added and the mixture was incubated at 37°C for 1 h (chicken pancreas conjugase) or 2 h (rat plasma conjugase).

2.3.1.3. Protocol (c). Extraction with trienzymatic treatment

The sample was weighed into a 100-ml conical flask. A 30-ml volume of 100 m*M* phosphate buffer (pH 6) containing 1% L-ascorbic acid, 25 mg of pronase and 50 mg of α-amylase was added and the mixture was incubated at 37°C for 4 h (in certain studies, α-amylase was omitted), then placed in a water bath at 100°C for 10 min. After being allowed to cool, it was adjusted to pH 7 with 5 *M* sodium hydroxide, then diluted to 50 ml with 100 m*M* phosphate buffer (pH 7) containing 1% L-ascorbic acid and centrifuged at 5000 rpm for 10 min. A 5–10 ml volume of the supernatant was placed in a 150 ml conical flask, then treated as described in protocol (b).

2.3.2. Precolumn conversion of folates to 5-CH_3-$H_4PteGlu_n$

A 5-ml volume of 100 m*M* phosphate buffer containing 40% sodium ascorbate (pH 7.4) was added to the preceding solution or to 500 μl of a folate standard (at approximately 15 to 80 nmol ml^{-1}). A 15-ml volume of 66 m*M* Tris buffer (pH 7.8), 1 ml of 2-octanol, then 10 ml of $NaBH_4$ at 120 g l^{-1} (approximately 32 mmol) were added. After shaking, the solution was left to stand for 10 min, then adjusted to pH 7.4 with 5 *M* acetic acid. An 80-μl volume of 37% formaldehyde (approximately 1 mmol) was added; after being shaken (less than 1 min), 10 ml of $NaBH_4$ at 120 g l^{-1} were added cautiously to the solution. The pH was then adjusted to a value lower than 1 with concentrated (37%) hydrochloric acid. The solution was left to stand for 10 min, then the pH was adjusted to pH 5 with 5 *M*

sodium hydroxide. A 10-ml volume of $NaBH_4$ at 120 g l^{-1} was once again added gently and the solution was left to stand for 20 min. The volume of the solution was finally made up to 100 ml with Tris buffer. The solution was filtered through a cellulose acetate filter (0.45 μm).

2.3.3. Purification of the sample on an affinity column

An affinity chromatography sorbent (Affi-gel 10) with immobilized folate binding proteins (FBPs), prepared as described by Konings [12], was used to purify the samples. The binding capacity of the FBP–Affi-gel 10 matrix (approximately 1 μg of folate) was tested periodically (after being used about five times) by overloading the column with a known amount of 5-CH_3-H_4PteGlu. Prior to use, the columns were washed with 10 ml of 100 m*M* phosphate buffer (pH 7).

A 10-ml (or more) volume of the sample previously obtained (Section 2.3.2) was loaded on to the column. The column was first washed with 10 ml of a 25 m*M* phosphate buffer solution (pH 7). The folates were then eluted with 8 ml of a solution containing 20 m*M* trifluoroacetic acid and 20 m*M* dithioerythritol in a 10-ml graduated flask containing 200 μl of a 25% solution of L-ascorbic acid and 40 μl of 600 g l^{-1} solution of potassium hydroxide. The final volume was adjusted to 10 ml with the eluent. The elution flow-rate was about 0.3 ml min^{-1}.

2.4. Chromatographic determination

2.4.1. Apparatus

The HPLC system consisted of a 3012 multisolvent delivery system (Varian, Les Ulis, France), a 9300 injection system (Varian), a 9050 UV–visible detector (Varian) and a 9075 fluorescence detector (Varian). Chromatographic peaks were quantified using a Star Chromatographic integrator (Varian).

A LiChrospher 100RP 18 endcapped (250×5 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) were used for all analyses.

2.4.2. Separation of folate monoglutamates

The mobile phase used was a gradient of acetonitrile and phosphate buffer (33 m*M*, pH 2.3) [7]. The starting eluent, a phosphate buffer–acetonitrile mixture (95:5, v/v), was applied for the first 8 min. The proportion of acetonitrile in the mixture was then increased linearly to 10% in 10 min, then to 20% in 7 min. The final composition of the mobile phase (80:20, v/v) was maintained for 5 min. The mobile phase was then immediately adjusted to its initial composition (phosphate buffer–acetonitrile, 95:5, v/v) and the elution was continued for 15 min in order to re-equilibrate the column. The flow-rate of the mobile phase was 1 ml min^{-1} and the injection volume 100 μl.

The fluorimetric detector operated at an excitation wavelength of 295 nm and at an emission wavelength of 356 nm. Detection by means of UV absorption was performed at 280 nm. Data were quantified using external calibration [the standards used were dissolved in a 100 m*M* phosphate buffer solution (pH 7) containing 1% L-ascorbic acid]. The standard deviations always referred to individual weighings.

For the recovery tests, a known quantity of a folate standard (approximately half of the quantity of folates present in the sample studied) was added before the extraction step (the recovery rates obtained were not taken into account for the quantification).

2.4.3. Separation of 5-CH_3-H_4PteGlu$_{1-8}$

The mobile phase used was a gradient of acetonitrile and phosphate buffer (50 m*M*, pH 4.6). The starting eluent was pure phosphate buffer. The proportion of acetonitrile was increased (linearly) to 10% in 15 min, then to 20% in 5 min. The final composition of the mobile phase (80:20, v/v) was maintained for 5 min. The mobile phase was then immediately adjusted to its initial composition and the elution was continued for 20 min in order to re-equilibrate the column. The flow-rate of the mobile phase was 0.8 ml min^{-1} and the injection volume 100 μl.

Fluorimetric detection, calibration and recovery tests were performed as above (Section 2.4.2).

3. Results and discussion

3.1. Conversion of folate standards to 5-CH_3-H_4PteGlu$_{1-8}$

Blair and Saunders [16] have prepared 5-CH_3-H_4PteGlu from PteGlu by reduction of this compound with sodium borohydride to H_4PteGlu, addition of formaldehyde to form 5,10-CH_2-H_4PteGlu and further reduction of the latter with sodium borohydride to 5-CH_3-H_4PteGlu. Moreover, according to Chanarin and Perry [17], 5-CH_3-H_4PteGlu may also be obtained from 5-CHO-H_4PteGlu by acidification and subsequent reduction of the 5,10-CH^+-H_4PteGlu formed with sodium borohydride. Finally, Brody et al. [18] have shown that it is possible to reduce 10-CHO-PteGlu to 10-CHO-H_4PteGlu with sodium borohydride under experimental conditions similar to those used by Blair and Saunders. This compound is then converted to 5-CH_3-H_4PteGlu according to the method of Chanarin and Perry.

In order to attempt to convert all of the folates possibly present in foodstuffs to 5-CH_3-H_4PteGlu, it seemed sensible to implement first the protocol of Blair and Saunders [16] and only in second place that of Chanarin and Perry [17], because the acidification of the reaction medium, essential in the second protocol to obtain the intermediate 5,10-CH^+-H_4PteGlu, would certainly cause partial destruction of the 5,10-CH_2-H_4PteGlu possibly present in the sample to be analyzed. However, it was necessary to adapt and optimize the different steps of the planned reaction scheme because the reactions presented above are usually not quantitative.

In view of the readily oxidizable nature of the folates, all chemical reactions performed on folate standards (or, subsequently, on food samples) were performed in a concentrated sodium ascorbate solution (1.8 *M* at the end of the various reactions performed). This antioxidant solution has in fact made it possible to obtain the highest conversion yields of the different folates under the experimental conditions ultimately selected. 2-Mercaptoethanol, very often combined with sodium ascorbate, in fact reacts with the formaldehyde used to carry out the addition of a one carbon unit to H_4PteGlu [22] and hence was not used.

By using a very large excess of sodium borohydride with respect to the quantity of folates to be converted (see Section 2.3.2), which required the addition of 2-octanol to the reaction medium in order to limit the production of foam, the reduction of PteGlu to H_4PteGlu (Fig. 1a, step 1) was not only quantitative but also immediate. This reaction was nonetheless allowed to proceed for 10 min in order to permit, in parallel, a partial conversion of 5-CHO-H_4PteGlu to H_4PteGlu (yield >60%; see below). Under these same conditions 5,10-CH_2-H_4PteGlu, 5,10-CH^+-H_4PteGlu and 10-CHO-H_4PteGlu were reduced quantitatively to 5-CH_3-H_4PteGlu (Fig. 1b, step 1). The excess of sodium borohydride was then destroyed by lowering the pH to 7.4. The optimal quantity of formaldehyde to be used for effecting the addition of a one carbon unit to the N^5 atom of H_4PteGlu was 80 μl (Fig. 1a, step 2). Under these conditions the conversion of H_4PteGlu into 5,10-CH_2-H_4PteGlu was complete and immediate. When larger quantities of reagent were used a decrease in the conversion yield was observed, probably owing to the attachment of a hydroxymethyl unit, not only to the N^5 nitrogen atom but also to the N^{10} nitrogen atom (according to the mechanism proposed by Kallen and Jencks [22]). This prevents the formation of 5,10-CH_2-H_4PteGlu. Owing to the instability of this chemical compound, its reduction to 5-CH_3-H_4PteGlu with sodium borohydride (Fig. 1a, step 3), which has already been demonstrated to be quantitative and instantaneous (see above), was achieved immediately after step 2. The application of the complete protocol (steps 1 to 3) to a solution of PteGlu has thus enabled 5-CH_3-H_4PteGlu to be obtained in excellent yield (95%). The conversion of H_2PteGlu and H_4PteGlu, other folates possibly present in the foodstuffs to 5-CH_3-H_4PteGlu and, moreover, intermediates formed in these conversions will hence be probably achieved in yields at least as satisfactory.

By employing only the protocol of Chanarin and Perry [17], it has never been possible to convert 5-CHO-H_4PteGlu to 5-CH_3-H_4PteGlu in a yield higher than 35% (Fig. 1c, steps 4 and 5). In fact the major chemical compound formed in this case (in a yield of 60–65%) was H_4PteGlu resulting from the reduction with sodium borohydride (step 5) of the 5-CHO-H_4PteGlu not converted to 5,10-CH^+-

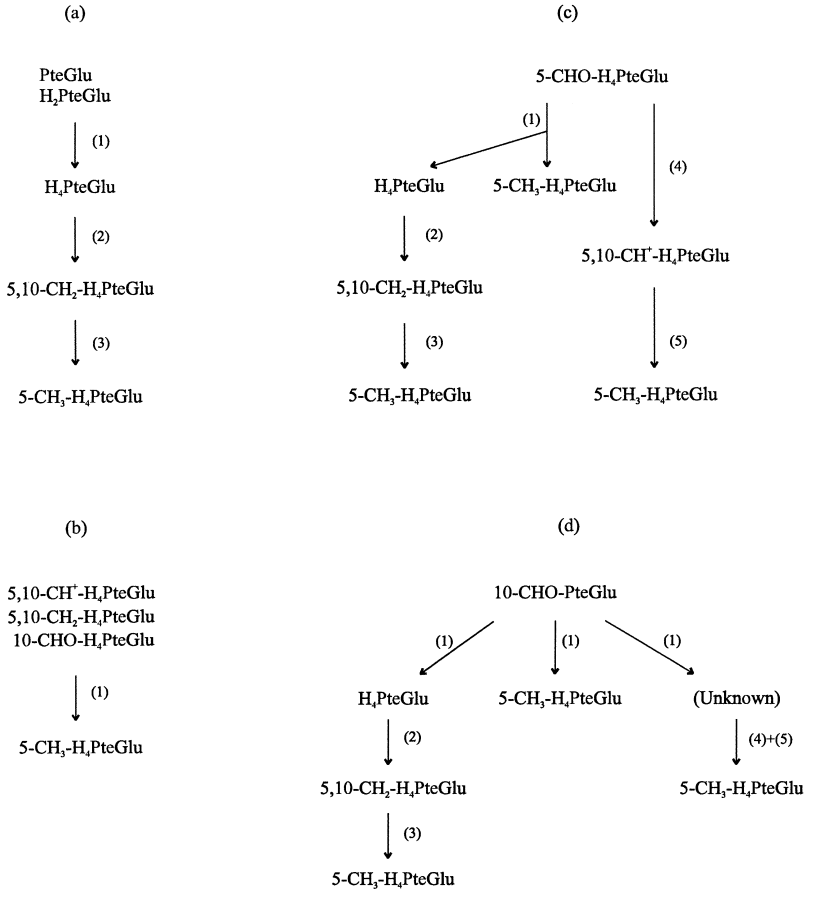

Fig. 1. Conversion of different folates to 5-CH_3-H_4PteGlu: (1) $NaBH_4$ (1.2 g), 10 min, pH 9.4; (2) 37% HCHO (80 μl), instantaneous, pH 7.4; (3) $NaBH_4$ (1.2 g), instantaneous; (4) concentrated HCl (pH<1), 10 min, addition of sodium hydroxide (pH 5); (5) $NaBH_4$ (1.2 g), 20 min.

H_4PteGlu on acid treatment (step 4). By submitting the solution of 5-CHO-H_4PteGlu to the entire protocol planned (Fig. 1c, steps 1 to 5), a high degree of conversion of this folate, of the order of 95%, to 5-CH_3-H_4PteGlu could be obtained, provided however, as specified above, that the sodium borohydride reaction at step 1 is allowed to proceed for at least 10 min, the H_4PteGlu formed under these conditions being then converted to 5-CH_3-H_4PteGlu (Fig. 1c, steps 2 and 3). This last folate could also be obtained directly by the reduction of 5-CHO-H_4PteGlu with sodium borohydride, but in very low yield (<5%).

As regards 10-CHO-PteGlu, its reduction by sodium borohydride (Fig. 1d, step 1) led mainly to the formation of H_4PteGlu (yield 55%), this folate then being converted quantitatively to 5-CH_3-H_4PteGlu by reaction with formaldehyde (step 2), then again with sodium borohydride (step 3). PteGlu, also detected when sodium borohydride had not been added in a sufficiently large excess, was formed without doubt as an intermediate. This reduction has also made it possible to obtain low quantities of 5-CH_3-H_4PteGlu (yield of the order of 5%). The yield of 5-CH_3-H_4PteGlu formed was thus about 60% after the completion of these first three steps. On the other hand, 10-CHO-H_4PteGlu was not found after step 1, in contrast to the findings of Brody et al. [18], very probably owing to its instantaneous conversion to 5-CH_3-H_4PteGlu in the presence of a large excess of sodium borohydride. The yield of

5-CH_3-H_4PteGlu formed at this step remained low (about 5%). However, the addition of the steps 4 and 5 to this protocol has enabled a conversion of 10-CHO-PteGlu to 5-CH_3-H_4PteGlu to be finally obtained in an acceptable yield of 75%. It was not possible to identify the nature of the folate that was converted in steps 4 and 5 (Fig. 1d).

The fact that a very satisfactory recovery of 5-CH_3-H_4PteGlu (92%) was obtained in the course of these various chemical reactions confirmed the stability of this folate.

The $PteGlu_{2-8}$ standards have also been subjected, each in turn, to this conversion protocol (steps 1 to 5). Chromatographic analyses have shown their complete disappearance each time after the third step and the appearance on the chromatogram of a single peak due to the formation of the corresponding 5-CH_3-$H_4$$PteGlu_n$. The separation of these different 5-CH_3-$H_4$$PteGlu_n$ could easily be achieved by reversed-phase chromatography using a 50 m*M* phosphate buffer (pH 4.6)–acetonitrile gradient as mobile phase (Fig. 2). The choice of this mobile phase and in particular that of the phosphate buffer has made it possible to attain for the detection of the different polyglutamate forms a much better sensitivity than that obtained by use of the mobile phase recommended by Cashmore et al. [21] [100 m*M* sodium acetate (pH 5.5)–acetonitrile gradient] (increase of a factor of 5- to 10-fold). The minimal detectable amounts of 5-CH_3-$H_4$$PteGlu_n$ obtained under these chromatographic conditions (Table 1) were very slightly lower than the values generally given in the literature for 5-CH_3-H_4PteGlu, which are comprised between 0.05 and 0.1 pmol [8,11,23], but were 10-fold higher than those obtained by Konings [12]. No explanation could be provided to justify such a difference. The detection limits of the other folates, which correspond to the mean values of the rather scattered available literature data [8,10–12,23], were, on the other hand and not surprisingly, markedly higher than that of 5-CH_3-H_4PteGlu. The precolumn chemical conversion of folates to 5-CH_3-H_4PteGlu hence confers excellent sensitivity on the chromato-

Table 1
Minimal detectable amounts of 5-CH_3-$H_4$$PteGlu_{1-8}$ [50 m*M* phosphate buffer (pH 4.6)–acetonitrile gradient as mobile phase] and other folates [33 m*M* phosphate buffer (pH 2.3)–acetonitrile gradient as mobile phase]

Folate	Limit of detection (pmol per injection)	
	UV[a]	Fluorescence[b]
5-CH_3-$H_4$$PteGlu_{1-8}$	2	0.02
H_4PteGlu	5	0.1
10-CHO-PteGlu	20	200
5-CHO-H_4PteGlu	2	1.5
PteGlu	2	–

[a] Absorption at 280 nm.
[b] Excitation at 295 nm and emission at 356 nm.

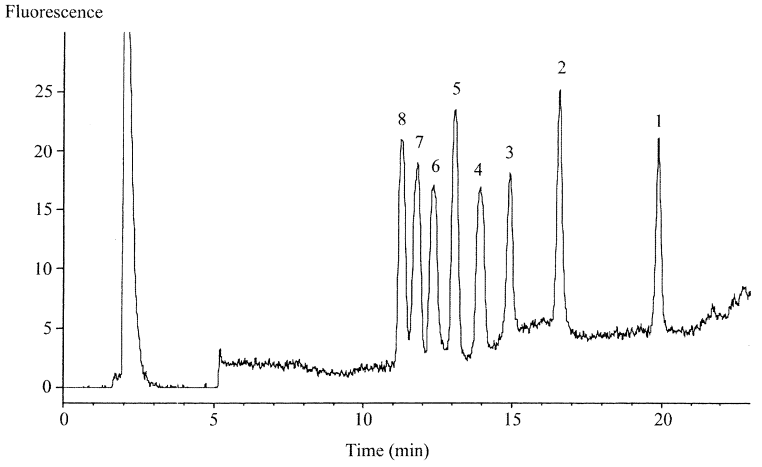

Fig. 2. Chromatographic separation of the various 5-CH_3-$H_4$$PteGlu_{1-8}$ (0.15 pmol of each folate) obtained after precolumn conversion of $PteGlu_{1-8}$ (the numbering of the different peaks refers to the number of glutamate groups in the folate concerned), using a 50 m*M* phosphate buffer (pH 4.6)–acetonitrile gradient as mobile phase and fluorescence detection at 356 nm (excitation at 295 nm).

graphic determination of the total folates. Moreover, folates in foodstuffs below their detection limit or small quantities of folates in foodstuffs where the major form is not 5-CH_3-H_4PteGlu can be determined.

3.2. Separation and analysis of the different folate polyglutamates (in the form 5-CH_3-H_4PteGlu$_n$) present in various food samples

The protocol drawn up for the conversion of folates to 5-CH_3-H_4PteGlu was first tested on samples of fruit juice, egg yolk and beef liver. These foodstuffs were selected on the one hand because the folates that they contain (PteGlu in fruit juice, 5-CH_3-H_4PteGlu in egg yolk, H_4PteGlu and 5-CH_3-H_4PteGlu in beef liver) were exclusively (in the case of fruit juice and egg yolk) or very predominantly (in the case of beef liver) in the monoglutamate form (see Table 4) and, on the other hand, because the chromatographic isolation of 5-CH_3-H_4PteGlu did not require prior purification of the sample on an affinity column. It was thus possible during the analysis of these food samples to make a full assessment of the efficiency of the chemical conversion proposed.

Such a precolumn conversion to 5-CH_3-H_4PteGlu of the PteGlu present in the fruit juice sample and of the H_4PteGlu present in the beef liver sample could be clearly demonstrated (Fig. 3). Moreover, whether there was a precolumn conversion or not, the overall contents of folate monoglutamates of beef liver and egg yolk did not vary (Table 2), thus confirming the quantitative nature of the conversion of H_4PteGlu to 5-CH_3-H_4PteGlu and the stability of 5-CH_3-H_4PteGlu during this operation. The determination in the fruit juice samples of a higher total folate content when a precolumn conversion was performed than

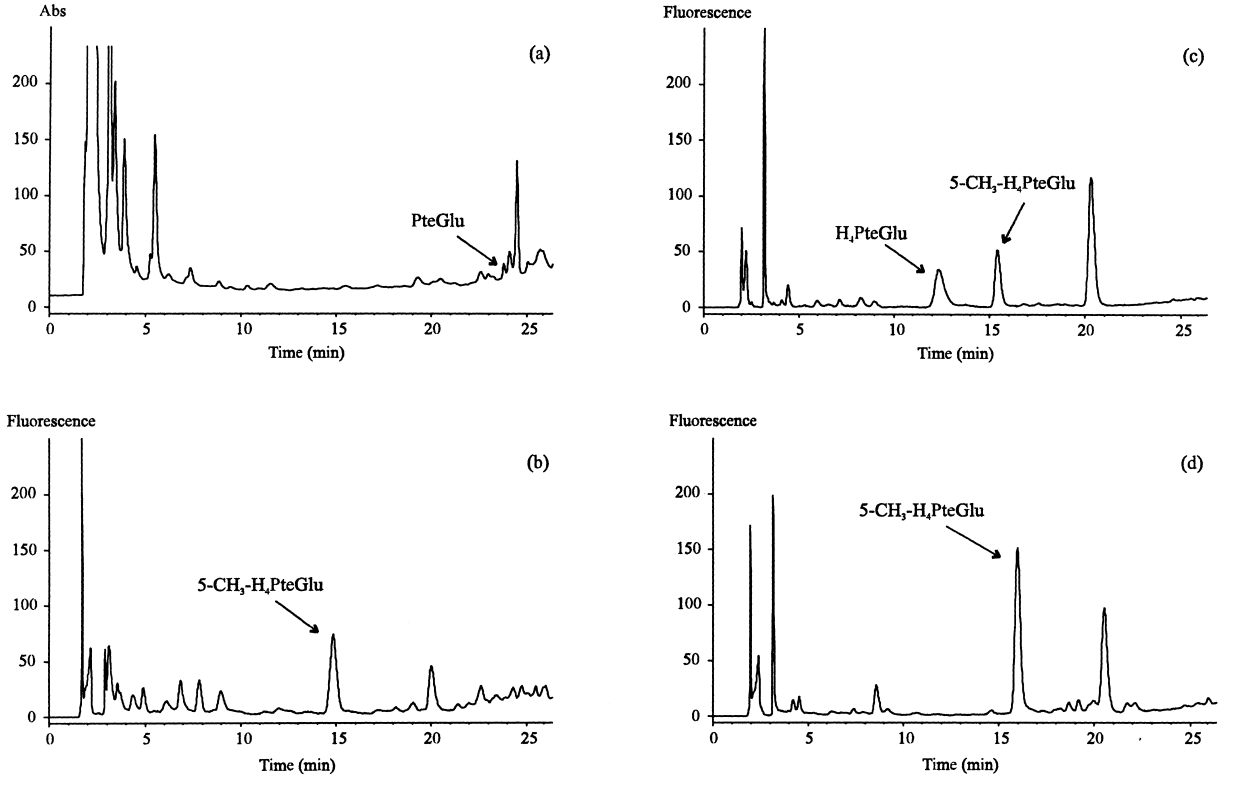

Fig. 3. Chromatographic separation of folate monoglutamates in fruit juice (a, b) and beef liver (c, d), without (a, c) or with (b, d) precolumn conversion [(a): UV detection at 280 nm; (b, c and d): fluorescence detection at 356 nm (excitation at 295 nm)], using a 33 m*M* phosphate buffer (pH 2.3)–acetonitrile gradient as mobile phase.

Table 2
Liquid chromatographic determination of folate monoglutamates and total folate contents (expressed in nmol g^{-1}) of beef liver, egg yolk and fruit juice samples obtained without (1) or with (2) precolumn conversion of folates into 5-CH_3-H_4PteGlu[a]

Food		Concentration			
		PteGlu	H_4PteGlu	5-CH_3-H_4PteGlu	Total folates
Beef liver	(1)	–	10.9 (1.1)	4.3 (0.2)	15.2 (1.2)
	(2)	–	–	15.5 (0.3)	15.5 (0.3)
Egg yolk	(1)	–	–	2.56 (0.02)	2.56 (0.02)
	(2)	–	–	2.69 (0.06)	2.69 (0.06)
Fruit juice	(1)	1.23 (0.07)	–	–	1.23 (0.07)
	(2)	–	–	1.68 (0.06)	1.68 (0.06)

[a] Average of three determinations (standard deviation in parentheses).

when it was omitted (Table 2) may result from an underestimation of the initial content of PteGlu owing to a lack of selectivity and sensitivity of the UV detector used to detect this non-fluorescent molecule (Fig. 3a) or to a possible conversion to 5-CH_3-H_4PteGlu of folates naturally present in the fruit juice and not detected by UV photometry [the presence of 5-CH_3-H_4PteGlu could actually be demonstrated in this fruit juice by carrying out detection by fluorimetry when a purification by means of affinity chromatography had been performed beforehand but the very low concentration found (0.10 nmol g^{-1}) did not permit a complete explanation of the deviation observed].

When this analytical method is applied to samples of beef liver and fruit juice, the recovery yields of PteGlu, 5-CHO-H_4PteGlu and 5-CH_3-H_4PteGlu, were always very satisfactory (Table 3). Those of 10-CHO-PteGlu were correct (Table 3) and very slightly higher than the conversion yield of this folate to 5-CH_3-H_4PteGlu when the reaction was performed in aqueous solution (see above).

Table 3
Recovery[a] of folate standards added to beef liver and fruit juice

Folate	Recovery (%)	
	Beef liver	Fruit juice
PteGlu	95 (1)	92 (5)
5-CH_3-H_4PteGlu	91 (2)	94 (3)
5-CHO-H_4PteGlu	98 (2)	93 (2)
10-CHO-PteGlu	83 (4)	79 (2)

Protocol used: extraction without enzymatic treatment, chemical conversion of folates to 5-CH_3-H_4PteGlu and fluorimetric quantification after liquid chromatographic isolation.

[a] Average of three determinations (standard deviation in parentheses).

All of the other foodstuffs studied contained considerable quantities of folate polyglutamates (n= 2 to 8), easily separated (in the 5-CH_3-H_4PteGlu$_{2-8}$ form) by liquid phase chromatography provided a precolumn purification of the extracts by affinity chromatography was used (Fig. 4). The summation of the different contents of 5-CH_3-H_4PteGlu$_n$ made it possible to obtain the total contents of folates of the different food samples analyzed (Table 4, line 1). The analytical results obtained for egg yolk and yeast are in very good agreement with those given by Seyoum and Selhub [24], which indicated the exclusive presence of 5-CH_3-H_4PteGlu in egg yolk at a content of 3.8 nmol g^{-1}, and the demonstration in yeast of hexa-, hepta- and octaglutamates at contents of 9.4, 38.0 and 8.0 nmol g^{-1}. In beef liver, on the other hand, these authors reported a total folate content markedly lower (9.7±0.7 nmol g^{-1}) than that obtained during this work. However, they were only able to detect folates monoglutamates whereas these latter are, in fact, a major (89%) but not unique constituent in this foodstuff (Table 4).

3.3. The enzymatic deconjugation treatment of the folate polyglutamates

If the objective of the analysis was to achieve a chromatographic separation of the different folates, deconjugation of the different polyglutamate forms

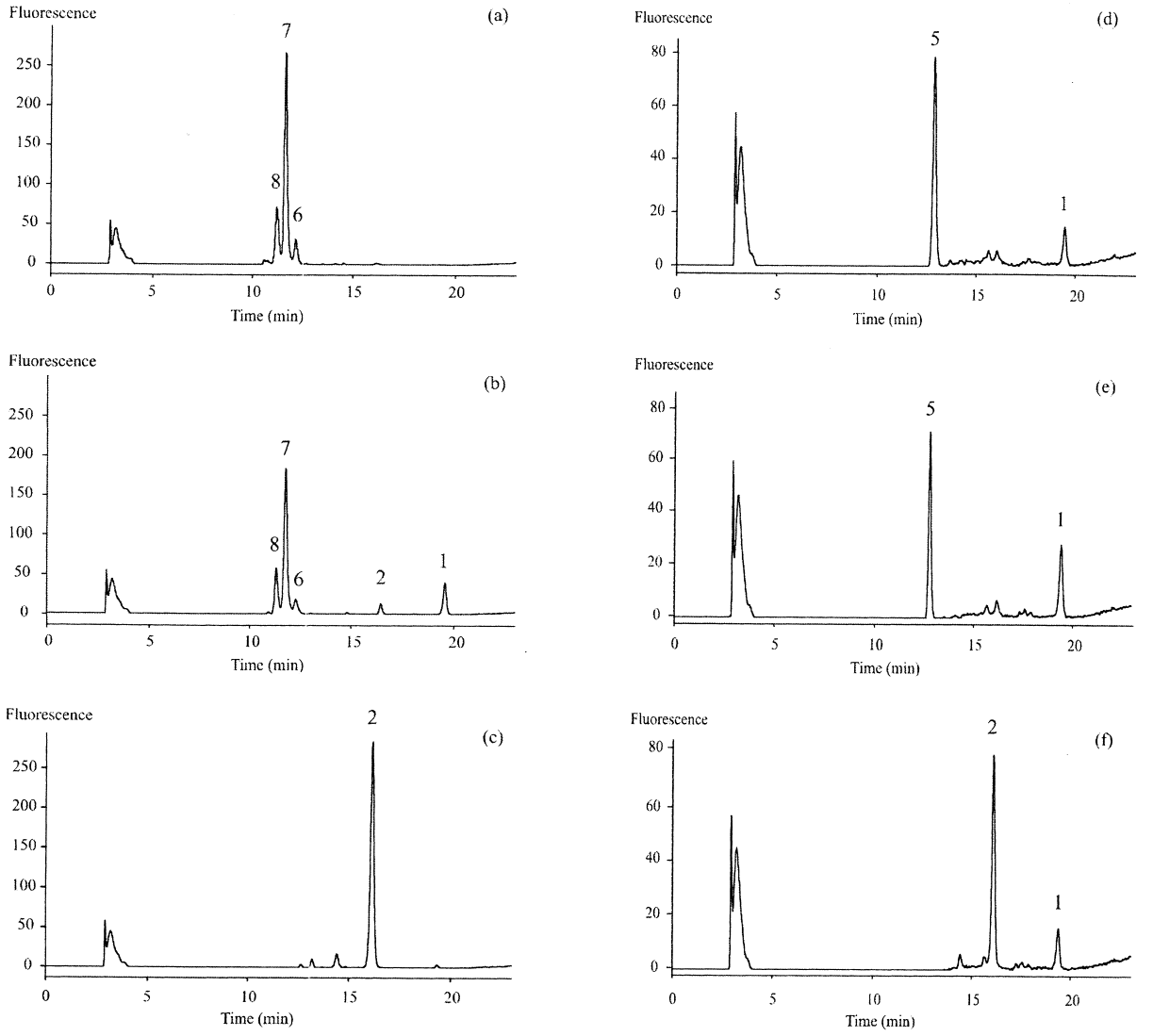

Fig. 4. Chromatographic separation of 5-CH_3-H_4PteGlu$_n$ in yeast (a, b, c) and peas (d, e, f) samples [no conjugase treatment (a, d); hydrolysis with rat plasma (b, e) or chicken pancreas (c, f) conjugase] (the numbering of the different peaks refers to the number of glutamate groups in the folate concerned), using a 50 m*M* phosphate buffer (pH 4.6)–acetonitrile gradient as mobile phase and a fluorescence detection at 356 nm (excitation at 295 nm).

would seem desirable. It is not in fact to be expected that the dietary folates, which comprise a multitude of compounds not only differing in pteroyl moiety but also differing in the number of attached glutamate units, can be successfully analyzed by liquid phase chromatography (difficulties of separation, lack of polyglutamate standards). In the analytical protocol proposed, in which a precolumn conversion of the different folate mono- and polyglutamates to 5-CH_3-H_4PteGlu$_n$ is performed, an analysis of the total folates can, on the contrary, be achieved without it being necessary to employ a deconjugation treatment (see Section 3.2). Inclusion of a deconjugation step will however simplify the chromatographic

Table 4
Liquid chromatographic determination of 5-CH_3-H_4PteGlu$_{1-8}$ and total folate concentration[a] (expressed in nmol g^{-1}) of various food samples obtained without (1) or after (2) sample treatment with chicken pancreas conjugase

Food		Concentration								
		Glu_8	Glu_7	Glu_6	Glu_5	Glu_4	Glu_3	Glu_2	Glu_1	Total folates
Yeast	(1)	12.8 (0.5)	31.8 (1.3)	5.5 (0.4)	–	–	–	–	–	50.1 (1.4)
	(2)	–	–	–	–	–	–	49.5 (1.4)	–	49.5 (1.4)
Beef liver	(1)	–	0.39 (0.05)	1.02 (0.09)	0.77 (0.08)	0.36 (0.10)	0.15 (0.03)	0.77 (0.12)	26.9 (1.9)	30.4 (1.9)
	(2)	–	–	–	–	–	–	3.50 (0.13)	24.6 (1.3)	28.1 (1.3)
Egg yolk	(1)	–	–	–	–	–	–	–	4.21 (0.01)	4.21 (0.01)
	(2)	–	–	–	–	–	–	–	4.00 (0.29)	4.00 (0.29)
Spinach	(1)	–	–	0.32 (0.01)	1.31 (0.02)	0.06 (0.01)	–	–	0.54 (0.01)	2.23 (0.03)
	(2)	–	–	–	–	–	–	1.73 (0.03)	0.54 (0.03)	2.27 (0.04)
Peas	(1)	–	–	–	1.10 (0.09)	–	–	–	0.35 (0.04)	1.45 (0.10)
	(2)	–	–	–	–	–	–	1.177 (0.007)	0.388 (0.008)	1.56 (0.11)
Wheat flour	(1)	–	–	0.20 (0.02)	0.313 (0.003)	0.06 (0.02)	–	–	0.179 (0.009)	0.75 (0.06)
	(2)	–	–	–	–	–	–	0.60 (0.03)	0.21 (0.02)	0.81 (0.04)
Powdered milk	(1)	0.035 (0.002)	0.046 (0.004)	0.056 (0.003)	0.037 (0.006)	0.020 (0.001)	–	–	0.41 (0.01)	0.60 (0.01)
	(2)	–	–	–	–	–	–	0.21 (0.02)	0.42 (0.02)	0.63 (0.03)
Beef fillet	(1)	0.014 (0.002)	0.015 (0.001)	–	–	–	–	0.022 (0.001)	0.094 (0.002)	0.144 (0.003)
	(2)	–	–	–	–	–	–	0.064 (0.004)	0.097 (0.006)	0.160 (0.007)
Apple	(1)	0.018 (0.001)	0.021 (0.001)	0.015 (0.001)	–	–	–	–	0.024 (0.002)	0.078 (0.003)
	(2)	–	–	–	–	–	–	0.073 (0.004)	0.021 (0.002)	0.095 (0.005)

[a] Average of three determinations (standard deviation in parentheses).

separation and will provide a more accurate determination of polyglutamates present in small quantities.

The chicken pancreas conjugase which has a pH optimum between 7 and 8.5 and which allows hydrolysis of the folate polyglutamates to the diglutamate stage [25] is the conjugase most often used in the microbiological assays [26–30]. When an analysis is performed by means of liquid chromatography, rat plasma conjugase, the pH optimum of which lies between 6.2 and 7.4, is usually preferred to it inasmuch as it seems to lead to deconjugation of the folates to the monoglutamate stage [10,12]. Another enzyme, hog kidney conjugase, has also sometimes been used [13,31]. The measurement of the activities of these various enzymes towards the PteGlu$_n$ standards has, however, shown that the hog kidney conjugase was markedly less efficient than the two other conjugases and that it was thus preferable not to select it (Table 5). When the substrates tested were food samples, the complete

Table 5
Activities of the different conjugases [measured at 37°C and pH 7 (rat plasma and chicken pancreas conjugases) or pH 4.5 (hog kidney conjugase)] expressed in percentage of PteGlu$_n$ hydrolysed to PteGlu$_2$ (for chicken pancreas conjugase) or PteGlu (for other conjugases)

Substrate	Conjugase		
	Rat plasma	Chicken pancreas	Hog kidney
PteGlu$_2$	98	–	55
PteGlu$_3$	85	86	59
PteGlu$_5$	83	85	73
PteGlu$_7$	83	92	85

hydrolysis of folate polyglutamates ($n>2$) to folate diglutamates by the chicken pancreas conjugase was still obtained, whereas the degree of hydrolysis of these polyglutamates to monoglutamates was very low when the rat plasma conjugase was used (Fig. 4). It did not exceed 25% in the samples of yeast and peas after an incubation for 2 h at 37°C. An increase of the time of incubation to 8 h did not lead to an improvement in the extent of hydrolysis. The use of rat plasma conjugase may thus lead to a considerable underestimation of the folate contents of a foodstuff if a large proportion of these compounds is in a polyglutamate form. The chicken pancreas conjugase was hence preferred to it even though its use has the inconvenience of requiring a separate analysis of the 5-CH_3-H_4PteGlu and 5-CH_3-H_4PteGlu$_2$, but this did not present analytical difficulties (Fig. 4). The total folate contents of the different foodstuffs studied, obtained with or without deconjugation treatment by means of the chicken pancreas conjugase, were moreover completely identical (Table 4).

The folate recovery yields obtained on analysis of a spinach sample by the analytical protocol thus proposed, including treatment with chicken pancreas conjugase, were quite satisfactory (Table 6), and in any case in conformity with what the conversion yields to 5-CH_3-H_4PteGlu of the different folate standards had led us to expect (see Section 3.1).

The day-to-day relative standard deviations for repeatability (four determinations in triplicate) of the measurement of total folates in samples of beef fillet (10%), peas (5%), spinach (7%) and beef liver (7%) were also satisfactory. The application of this protocol to the analysis of two reference certified materials [pig's liver (CRM 487) and milk powder (CRM 421)] has moreover enabled a folate content [(31.8±0.8) nmol g^{-1}] to be obtained for the first of these foodstuffs within the confidence interval of the certified value [(30.15±2.95) nmol g^{-1}]. As for the folate content obtained for milk powder [(2.4±0.1) nmol g^{-1}], it proved to be lower than the value certified by microbiological assay [(3.22±0.32) nmol g^{-1}] but of the same order of magnitude as that obtained by Konings [12] [(2.43±0.04) nmol g^{-1}] using a chromatographic method (with separate determination of the different folate monoglutamates).

Table 6
Recovery[a] of folate standards added to spinach samples

Folate	Recovery (%)
PteGlu	85 (6)
PteGlu$_2$	93 (4)
5-CH_3-H_4PteGlu	92 (5)
5-CHO-H_4PteGlu	98 (7)
10-CHO-PteGlu	78 (5)

Protocol used: chicken pancreas conjugase treatment, chemical conversion of folates to 5-CH_3-H_4PteGlu$_{1-2}$, affinity chromatographic purification and fluorimetric quantification after liquid chromatographic isolation.

[a] Average of four determinations (standard deviation in parentheses).

3.4. The need to subject the food samples to prior treatments with protease and amylase

On performing the microbiological analysis of folates in various foodstuffs, Martin et al. [27] sometimes noted a considerable increase in the contents of these vitamins (up to 50%) when a protease and an α-amylase were combined with the conjugase and they attributed this to more efficient extraction of the folates which are assumed to be linked to the food matrix. Many workers have been able to confirm this result, although not on all of the foodstuffs analyzed, whether the analysis was microbiological [28–30,32,33] or chromatographic [10,12]. The different results published are moreover not always consistent: whereas Shresta et al. [30] observed no effect of protease and amylase hydrolyses on the folate content of spinach, Martin et al. [27] obtained an increase of 21% in the content of this same foodstuff after a trienzymatic treatment. Aiso and Tamura [32] determined the increase in this same case to be 51%.

The results obtained in this study have consistently shown that treatment with pronase (25 mg) or with a mixture of pronase (25 mg)/α-amylase (50 mg) had no effect on the contents of folates of five different foodstuffs (Table 7). The trienzymatic treatment therefore does not seem to be essential, at least not to the application of the analytical protocol suggested. However, this observation requires confirmation by

Table 7
Influence of the extraction protocol on folate concentrations[a] in various foodstuffs

Extraction protocol	Concentration (nmol g^{-1})				
	Yeast	Spinach	Beef liver	Beef fillet	Peas
(1)	54 (3)	1.85 (0.01)	22.8 (1.3)	0.12 (0.01)	1.53 (0.06)
(2)	50 (2)	1.80 (0.10)	20.9 (0.7)	0.10 (0.01)	1.58 (0.03)
(3)	50 (4)	1.95 (0.07)	20.1 (1.1)	0.13 (0.02)	1.49 (0.11)

(1) Chicken pancreas conjugase (5 mg). (2) Pronase (25 mg) and chicken pancreas conjugase (5 mg). (3) Pronase (25 mg), α-amylase (50 mg) and chicken pancreas conjugase (5 mg).
[a] Average of three determinations (standard deviation in parentheses).

extension to the analysis of a larger number of foodstuffs.

4. Conclusion

The implementation of a precolumn conversion of folates (in the mono- and polyglutamate forms) to 5-CH_3-H_4PteGlu$_n$ in a chromatographic protocol does not of course allow the identity of the pteroyl moiety of the folates present in foods to be determined. If one admit that the various folate monoglutamates have bioactivities very different from one another, which is not readily apparent from reading the articles devoted to this problem [34–36], this might certainly appear to be a disadvantage of the suggested analytical protocol. This protocol, has, however, made it possible to achieve a chromatographic separation of the different 5-CH_3-H_4PteGlu$_{1-8}$ and to obtain a very sensitive and specific determination of the total folate content of the foodstuff studied (characteristics which the chromatographic methods usually recommended do not possess). The change in the chromatograms when a deconjugation treatment was employed initially showed, as anticipated, that the chicken pancreas conjugase led to complete deconjugation of the folate polyglutamates ($n>2$) to folate diglutamates, whereas the rat plasma conjugase only converted these polyglutamate ($n>1$) forms very partially to monoglutamate forms (this deconjugation was, conversely, complete when the substrate was a PteGlu$_n$ standard). The prior treatment the foodstuff samples with a protease and an α-amylase for the purpose of releasing folates possibly linked to proteins and polysaccharides has been demonstrated to be unnecessary during this study.

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