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Determination of folate vitamers in food and in Italian reference diet by high-performance liquid chromatography ☆

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Abstract

A trienzyme treatment (conjugase, α-amylase, protease) followed by affinity chromatography and reversed-phase HPLC with UV and fluorescence detection was performed for the quantification of folate vitamers in legumes (chickpea and beans), processed meats (salami Milano and Parma ham) and in an Italian reference diet. This method allowed a good separation of six folate vitamers: 5-methyltetrahydrofolate, 5-formyltetrahydrofolate, folic acid, 10-formylfolic acid, 10-formyldihydrofolate and tetrahydrofolate within 30 min. Recovery, reproducibility and limits of detection of the method are reported. HPLC results were 24–52% lower than the microbiological assay findings.

Keywords: Food analysis; Legumes; Processed meats; Diets; Folate vitamers

1. Introduction

Folate is a B group vitamin acting as a coenzyme in several single carbon transfer reactions to synthesize DNA, RNA and protein components. Folate is the generic term to indicate compounds naturally occurring in food that have vitamin activity similar to pteroylmonoglutamic acid (folic acid), that is the synthetic product.

The terms ‘folic acid’ and ‘folate’ are often used interchangeably, but folic acid is approximately twice as bioavailable as the folate naturally occurring in food [1]. Most folates in food occur as polyglutamates. The predominant natural form of folate in food, both in fruits and vegetables, is 5-methyltetrahydrofolate (5-CH_3-H_4folate) [2], in animal products are 5-CH_3-H_4folate and tetrahydrofolate (H_4folate) [3], in cereal products also 5-formyltetrahydrofolate (5-HCO-H_4folate), 10-formyl-H_2folate and 10-formyl-folic acid have been detected in reasonable amounts, together with 5-CH_3-H_4folate and folic acid [4].

This work presents the application of several procedures including a modified trienzyme extraction, affinity chromatography, and reversed-phase HPLC with UV and fluorescence detection for the

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quantification of the most abundant forms of folate [4,5].

Specific kinds of food were selected from vegetables (chickpea and beans), processed meats (salami and ham) and a whole diet corresponding to Italian national intakes (Italian reference diet) were selected. HPLC data are compared with the results obtained using the microbiological AOAC method modified by Tamura [6].

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Methanol and acetonitrile were purchased from Merck (Darmstadt, Germany).

2.2. Standards

Tetrahydrofolic acid trihydrochloride (H_4folate), 5-methyltetrahydrofolate (5-CH_3-H_4folate calcium salt), 5-formyltetrahydrofolate (5-HCO-H_4folate calcium salt), folic acid, pteroyltri-L-glutamic acid ($PteGlu_3$), 10-formylfolic acid (10-HCO-folic acid), 5,10-methylenetetrahydrofolic acid, magnesium salt and 5,10-methenyltetrahydrofolic acid hydrochloride were obtained from Dr. Schirck's Labs. (Jona, Switzerland). 10-HCO-H_2folate was produced from 5,10-methenyltetrahydrofolate as described by Pfeiffer et al. [4]. Standards were dissolved as described by van den Berg et al. [7] and purities calculated using molar extinction coefficients at pH 7.0 [8]. Standard solutions were stored in 0.01 *M* acetate buffer (pH 4.9) with 1% ascorbate at −18°C.

2.3. Sampling

Dry seeds (100 g) of chickpea (*Cicer arietinum* L.) and common bean (*Phaseolus vulgaris* L.) var. Borlotto cultivated in Italy were soaked for 18 h in distilled water, drained and cooked with 1000 ml distilled water until the optimal cooking firmness was reached (after 1 h).

The Italian reference diet was formulated on the basis of the food intake collected in the National Food Consumption Survey conducted by the National Institute of Nutrition [9]. All food representative of daily intake were mixed and homogenized. Legume and diet samples were lyophilized before the analysis and stored frozen (−18°C).

Samples (100 g) of salami Milano and Parma ham were purchased from retail stores and supermarkets (seven in total) in the city of Rome and pooled in equal portions of each.

2.4. Sample extraction

Processed meat samples (salami and Parma ham) were weighed in duplicate (5 g/50 ml final volume) and for lyophilized samples (chickpea, beans, diet) 2.5 g was dissolved to a final volume of 50 ml. Samples were extracted with about 35 ml of boiling extraction buffer. Lyophilized samples were extracted with 2-(N-cyclohexylamino)ethane sulfonic acid (CHES)–4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES) buffer, pH 7.85, containing 2% (w/v) sodium ascorbate and 10 m*M* 2-mercaptoethanol [4].

Salami and Parma ham were extracted with 0.1 *M* phosphate buffer, pH 6.0, containing 1% (w/v) sodium ascorbate and 0.1% 2-mercaptoethanol [5]. This buffer extraction was preferred for meat samples because it gave reproducible results in previous studies [3,10].

After adding the hot buffer and flushing the samples with nitrogen the sample tubes were capped and heated for 10 min in a boiling water bath. Cooled samples were then homogenized with an Ultra Turrax for 30 s at a speed of 13 500 rpm, and filled to volume with the extraction buffer.

2.5. Sample deconjugation

Samples (25 ml) were deconjugated at pH 4.9 with 1 ml hog kidney conjugase prepared according to Gregory et al. [11] for 3 h at 37°C under a nitrogen atmosphere in a shaking waterbath. The deconjugation was carried out in duplicates. The deconjugation efficiency was determined by adding pteroyltriglutamate (Pte Glu_3) in excess into one of the sample mixtures of each food. This extract was filtered after incubation and heat denaturation and injected without any purification. Conversion of 90% (as peak areas) of pteroyltriglutamic acid into

pteroylglutamic acid in the sample matrix was considered adequate. By this procedure the presence of strong conjugase inhibitors was excluded, because the added triglutamate was deconjugated successfully in all sample matrices.

Together with conjugase, 1 ml (20 mg/ml) α-amylase (A-6211 Sigma, St. Louis, MO, USA) was added to the diet and chickpea and bean samples (25 ml). After 3 h, 2 ml protease (P-5147, Sigma) (2 mg/ml) was added to all incubated samples (25 ml) after bringing the pH back to 7.0 with sodium hydroxide. The protease was allowed to act for 1 h at 37°C before boiling the samples for 10 min in order to inactivate the enzymes. This mixture was centrifuged for 15 min at 11 950 *g* (Sorvall SS-34 rotor) and the pellet resuspended into a small volume of 0.1 *M* phosphate buffer containing 0.2% ascorbate and centrifuged.

2.6. Sample purification

The sample extract was applied to an affinity column (1.8 ml) prepared in the laboratory from Affigel 10 (Bio-Rad Laboratories, Richmond, CA, USA) and folate binding protein (Scripps, CA, USA) [4]. The covalenty bound folate-binding protein (FBP) selectively binds folate compounds at pH close to neutrality. The purified folates were eluted with very low pH using trifluoroacetic acid [4] and injected into the HPLC system.

The FBP-affinity columns had a binding capacity >3 μg of folic acid. Satisfactory standard and sample recoveries (range: 78–113%) were obtained when the columns were loaded with <1 μg of a mixture of folate derivatives.

2.7. HPLC analysis

A Varian Vista 5500 liquid chromatograph was equipped with a cooled Waters WISP 700 autosampler and Waters 470 fluorescence and Waters 487 dual wavelength absorbance detectors (Waters, Milford, MA, USA). A data acquisition system (MILLENNIUM chromatography manager) was used to collect chromatographic data and to evaluate peak areas.

Folate monoglutamates were separated using a Shandon (Cheshire, UK) Hypersil ODS column (150×4.6 mm, 3 μm). Gradient elution was performed with acetonitrile–30 m*M* phosphate buffer, pH 2.2, and the flow-rate was 0.8 ml/min. The run time was 30 min. The gradient started at 5% (v/v) acetonitrile which was maintained isocratically for the first 9 min, thereafter the acetonitrile concentration was raised linearly to 17% (v/v) within 30 min. The injection volume was 100 μl.

The absorbance of all eluted folates was monitored with an UV detector set at 290 nm, and with a fluorescence detector set at 290 nm excitation and 356 nm emission wavelengths (for reduced folate monoglutamates). Additionally, an excitation wavelength of 360 nm and emission wavelength of 460 nm were used for 10-formylfolic acid. Peak identification was based on the retention time, spiking and comparison of the ratios of fluorescence and UV peaks with that of the standard compounds.

2.8. Quantification and recovery studies

Quantification was based on an external standard method in which peak area was plotted against concentration. Calibration plots using least-squares regression analysis for each compound were prepared every day. The calibrants were purified through FBP-containing affigel columns. Four levels of calibrants were purified and the calibration curve was prepared from those by using similar standard levels as expected in the samples.

A standard solution of all folate derivatives found in the samples was used for recovery studies to assess the loss of folates during affinity chromatography. Spiking (addition of standard compounds into the purified sample extract) was also performed to increase the reliability of peak identification but was not considered adequate on its own. 5-CH_3-H_4folate, 10-HCO-PGA and H_4folate quantification was based on fluorescence detection, 5-HCO-H_4folate, PGA and 10-HCO-H_2folate were quantified by UV.

2.9. Microbiological assay

The total folate content of deconjugated samples was also determined by microbiological assay with *Lactobacillus casei* [6] using folic acid casei medium (Difco, Detroit, MI, USA). Portions of the extracts were flushed with nitrogen and stored at −20°C until analysis.

Table 1
Minimum detectable amounts of folate vitamers

Vitamin	Limit of detection (ng per injection)	
	UV	Fluorescence
H_4folate[a,b]	0.6	0.08
5-CH_3-H_4folate[a,b]	0.4	0.04
10-HCO-H_2folate[a]	0.8	–
10-HCO-PGA[a,c]	0.7	0.1
5-HCO-H_4folate[a,b]	0.6	0.6
PGA (folic acid)[a]	0.2	–

[a] Ultraviolet detection at 290 nm.
[b] Fluorescence at excitation 290 nm, emission 356 nm.
[c] Fluorescence at excitation 360 nm, emission 460 nm.

A certified reference sample (mixed vegetables) furnished by Institute of Food Research (Norwich, UK) was analysed as a check on the accuracy of the analysis. Experimental values of certified sample folate content (312±29.2 μg/100 g dry mass) obtained with the microbiological assay were not statistically different from the reference value (314±14.5 μg/100 g dry mass) [12].

3. Results and discussion

Detection limits in this HPLC system (defined as signal three times the height of the noise level, except for 5-HCO-H_4 folate for which it was twice the noise level) are shown in Table 1.

The recoveries of folate standards added to salami carried through affinity chromatography purification are presented in Table 2. Recoveries of the six vitamers added to salami ranged from 78–113% and these values obtained after affinity chromatography are in the same range as those obtained after anion-exchange purification [2]. Affinity chromatography with FBP has been successfully used for the determination of folate polyglutamate chain lengths in several kinds of food by Selhub and co-workers [13,14]. Pfeiffer et al. [4] analysed folates as their monoglutamates in cereal foods combining affinity and reversed-phase liquid chromatography. Our findings support the results of Pfeiffer et al. [4] in that affinity chromatography using immobilized FBP provided a very specific and effective purification along with a concentration of extracts. Although the columns were expensive and tedious to prepare, they were very stable and lasted for several months in weekly use.

Table 2
Recoveries of folate monoglutamates added to food sample (salami) after affinity chromatographic purification

Folate	Amount added (ng/100 g)	Recovery (%)[a] (X±S.D.)
H_4folate	60	109±13
5-CH_3-H_4folate	60	86±3
10-HCO-H_2folate	202	106±12
10-HCO-PGA	60	102±10
5-HCO-H_4folate	60	78
PGA (folic acid)	120	113±9

[a] Each value represents the mean of three determinations±S.D.

Calibration curves (Figs. 1 and 2) showed a linear response of peak area versus concentration of folate vitamers both for UV and fluorescence detection. During method validation it was noted that the best practise was to take the calibrants through FBP-affinity purification prior to the establishment of calibration curves. Particularly the stability, and therefore the recoveries, of H_4folate were low for the lowest levels of this calibrant. Due to the low binding capacity of 5-HCO-H_4folate to the FBP, the columns were never loaded with more than 1 μg of total folates. The retention times were repeatable with RSD<2%, n=4 injections.

Fig. 3a–c shows the chromatograms of a chickpea sample with UV detection at 290, with fluorescence detection at 290 nm (excitation), 356 (emission), with fluorescence at 360 nm (excitation), 460 nm (emission), respectively. The adapted gradient HPLC method allowed a good separation of folate monoglutamates within 30 min. Using UV (Fig. 3a) H_4folate, 5-CH_3-H_4folate, 10-HCO-H_2folate, 10-HCO-PGA, 5-HCO-H_4folate and folic acid (PGA) were detected. Using fluorescence detection at 290 excitation, 356 emission (Fig. 3b) H_4folate, 5-CH_3-H_4folate and 5-HCO-H_4folate were detected Then, folic acid and 10-HCO-H_2folate could only be detected using UV detection (Fig. 3a).

For 5-HCO-H_4folate there were frequently some interfering impurities using fluorescence, therefore detection and quantification using UV was necessary.

In chickpea (Fig. 3a) and also in the bean sample it was noted that even if the intensity of the peak

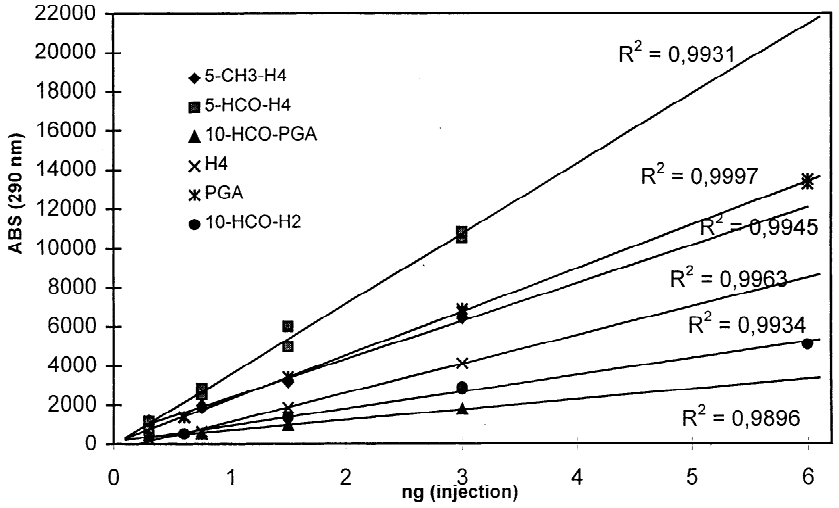

Fig. 1. UV calibration curves for folate vitamers.

eluting at 26 min (as 10-HCO-PGA) with UV detection was increased by spiking, when specific wavelengths for this vitamer (fluorescence excitation 360/emission 460) were applied (Fig. 3c) the assumption of rather large quantities of 10-HCO-PGA present in these particular samples had to be discarded. This interference was not noted for the other samples examined.

Chickpea (Fig. 3a and b) and bean samples also showed other unknown peaks with similar retention values, but their identities were not ascertained. The presence of these unknown peaks even after folate specific purification (with folate-binding protein) was rather unexpected. However, the identification of these peaks was outside the scope of this study. Pfeiffer et al. [4] in fortified and unfortified white breads also found non identified derivative/components retained by affinity chromatography. The

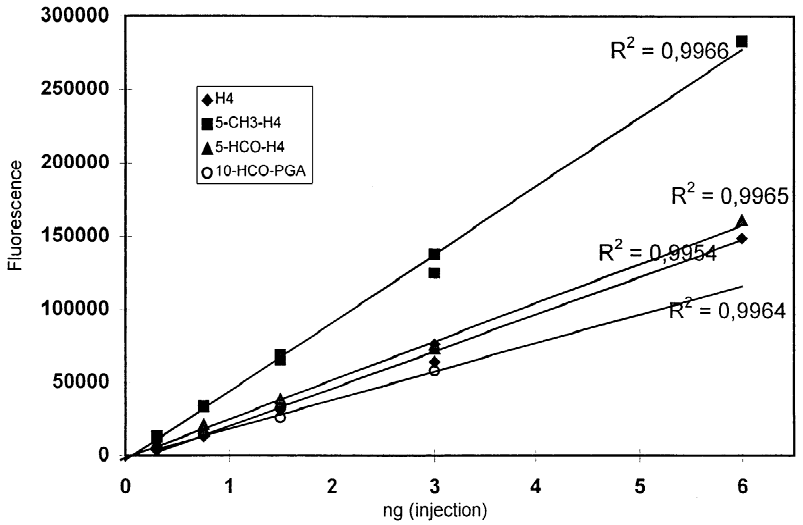

Fig. 2. Calibration curves for fluorescent folate vitamers. H_4-, 5-CH_3-H_4- and 5-HCO-H_4folates were detected at 290 nm (excitation) and 356 (emission); 10-HCO-PGA was detected at 360 nm (excitation) and 460 nm (emission).

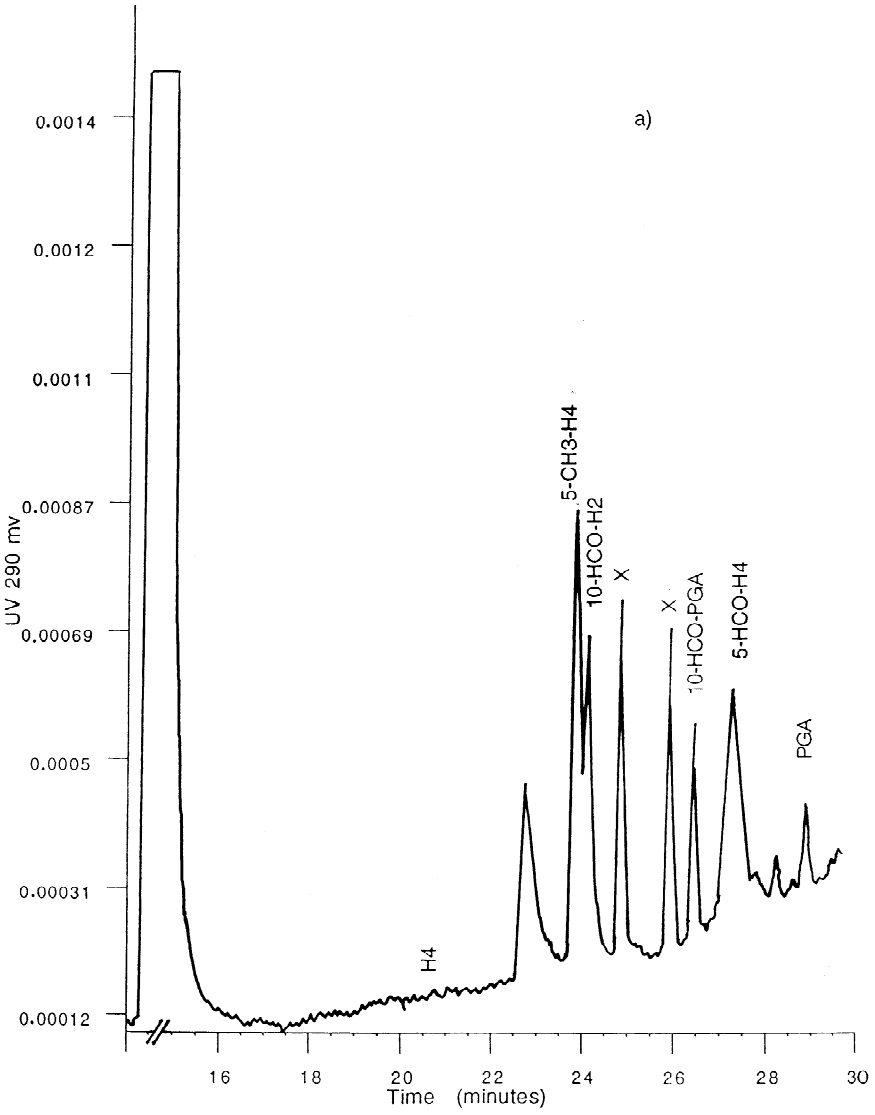

Fig. 3. Reversed-phase chromatograms of the main folate forms present in cooked chickpeas: (a) UV detection at 290 nm (b) fluorescence detection at excitation 290 nm and emission 356 nm (c) fluorescence detection at excitation 360 nm and emission 460 nm. A Shandon Hypersil column (150×4.6 mm, 3 μm) and a mobile phase of 30 m*M* acetonitrile–phosphate buffer, pH 2.2, with increasing acetonitrile concentration were used.

presence of these peaks does, however, highlight the necessity of careful peak identification and verification in HPLC work. It also shows, in our case, that fluorescence detection may not always be more specific, or more reliable.

The folate vitamer content obtained by HPLC and the total folate content obtained by a microbiological method are shown in Table 3. The most abundant vitamers in the Italian diet were 10-HCO-PGA, 5-HCO-H_4folate and 5-CH_3-H_4folate, in chickpeas 10-HCO-H_2folate, in beans and in salami 5-HCO-H_4folate. Small amounts of folic acid were detected in all samples. In Parma ham the folate vitamers were below quantification limits.

The total folate contents determined by HPLC were 24–52% lower than the folate contents de-

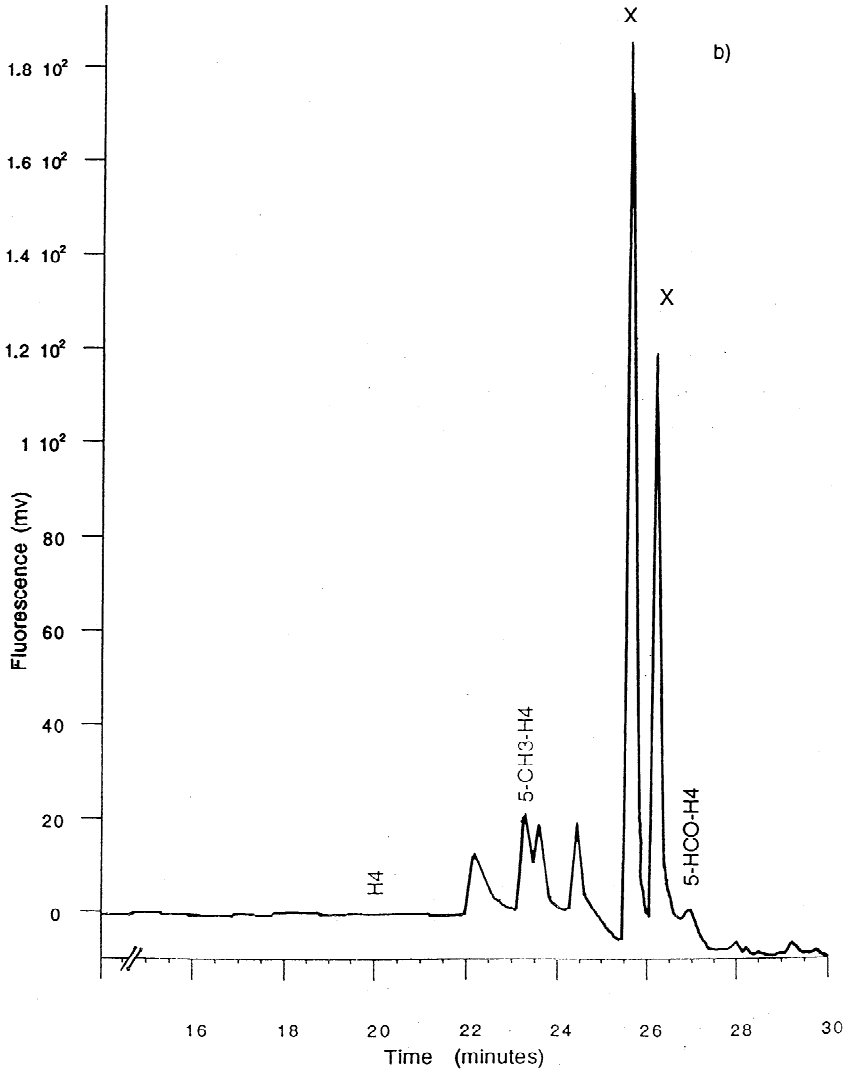

Fig. 3. (*continued*)

termined by the microbiological assay; in chickpeas and beans the sum of vitamers is much lower than that determined by the microbiological assay. This could be due to the presence of other compounds with folate activity in these food samples as indicated by the chromatograms.

At present there are only very few works comparing HPLC and microbiological results for folates [4,12,15]. Pfeiffer et al. [4] found very good agreement between the two methods when examining cereal products. On the contrary, Finglas et al. [12] discussing the results of five folate intercomparison studies on four reference samples, concluded that certified values of total folate content were obtained by microbiological assay for all reference samples. But, as regards the HPLC method, indicative values could only be proposed for 5-methyl tetrahydrofolate. The necessity of further work for HPLC analysis of folates was stressed.

However, taking into account the microbiological data, folate levels in the Italian reference diet satisfy the recommended daily intake for folate [16]. Chic-

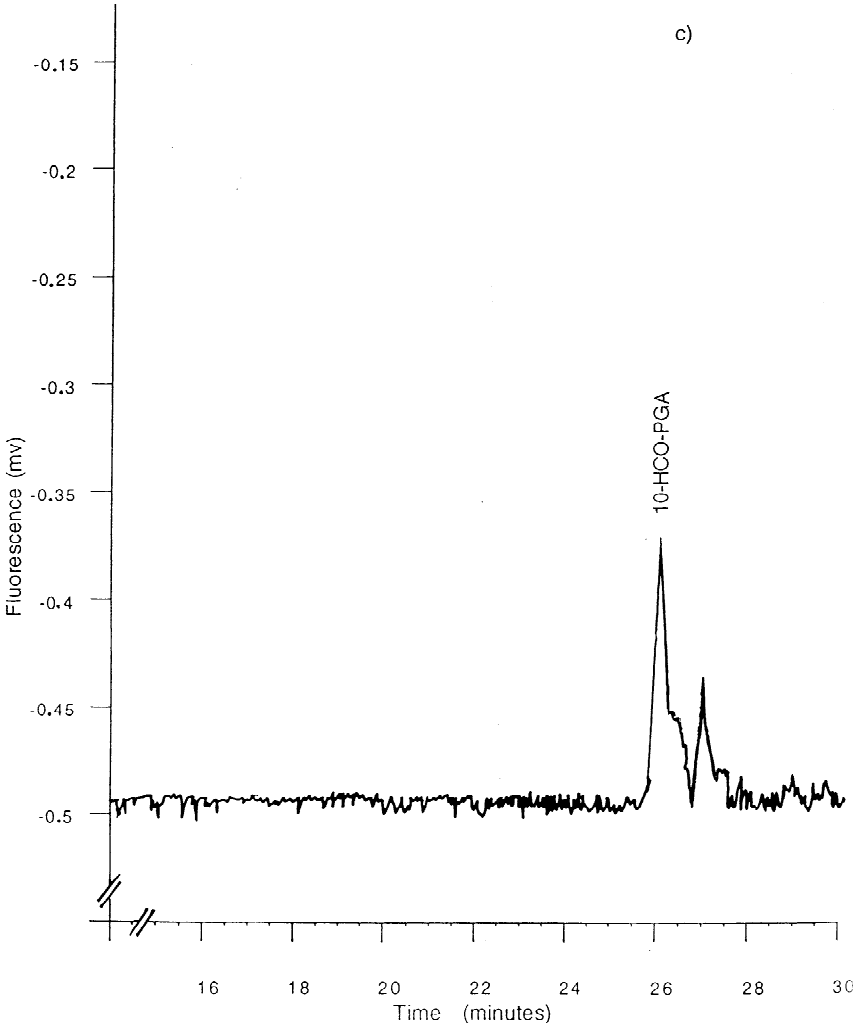

Fig. 3. (*continued*)

kpeas and beans were confirmed to be good sources of folate; whereas salami showed very low folate levels.

4. Conclusions

The method described (trienzyme deconjugation, FBP purification, HPLC determination by UV and fluorescence detectors) allows a good separation of the main folate vitamers in food samples. Both UV and fluorescence analyses should be used to achieve more reliable results. This method can be applied to different food products: both animal and vegetable products, along with very complex matrices such as diet samples. The microbiological and HPLC results are not always in agreement, probably due to the presence of other compounds with folate activity in beans and chickpeas. We therefore stress the necessity for a careful peak identification in the HPLC analysis of food folates in order to produce reliable results.

Table 3
Folate vitamers in food samples (μg/100 g fresh mass)[a]

	5-CH_3-H_4 folate	5-HCO-H_4 folate	PGA	10-HCO-PGA	10-HCO-H_2 folate	H_4folate	Sum of vitamer[b]	Total folate microbiological assay (μg/100 g)
Italian National reference diet	1.7±0.02	1.8±0.09	0.4±0.28	2.4±0.25	0.6±0.28	n.d.	6.9	9.9±0.25
Traditional cooked chickpeas	9.1±0.57	6.1±0.59	1.0±0.08	2.3±0.12	15.7±0.62	n.d.	34.2	44.8±2.27
Traditional cooked beans	4.7±0.23	7.5±0.7	2.4±0.19	1.4±0.14	n.d.	2.5±0.6	18.5	38.8±3.10
Salami Milano	0.7±0.06	3.3±0.04	0.9±0.14	0.9±0.13	n.d.	0.7±0.07	5.8	9.6±1.00
Ham, Parma	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		1.8±0.14

[a] Each value represents the mean ±S.D. of three determinations for HPLC, and of four determinations for microbiological assay.
[b] Sum as individual.
n.d.=not detectable<detection limit.

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