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Analytical Methods Section

First BCR†-intercomparison on the determination of folates in food

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The first BCR intercomparison on the determination of folate in food was designed to study the state-of-the-art in folate analysis in a group of experienced vitamin laboratories in Europe. In all 15 participants from 8 countries took part using microbiological and HPLC procedures, enzyme protein-binding assays (EPBA) and commercial radioprotein-binding assay kits (RPBA). The participants were asked to quantify folate levels in a lyophilised Brussels sprout material, which had been specifically prepared as a candidate reference material for vitamin work, using their preferred method of analysis. Three types of deconjugation were also investigated, human plasma (HP), chicken pancreas (CP) and hog kidney (HK). Generally good agreement was obtained by participants using the microbiological procedures. Folate levels determined after CP deconjugate treatment (mean = 984 μg per 100 g dry matter, SD = 237, n = 6) were 19% higher than those levels found after HP deconjugation (mean = 824 μg per 100 g dry matter, SD = 147, n = 6). The use of autoclaving followed by deconjugation with either HP or CP enzymes gave lower (10–20%) folate levels (determined by microbiological assay) when compared to refluxing and deconjugation with the same enzymes. Hog kidney deconjugase enzyme and autoclaving/refluxing was not as effective. Although the HPLC results from the 2 participants who were able to complete the study agree reasonably well with the microbiological data, there were differences in the proportion of the individual folate forms measured. One participant found 5-CH_3THF (55%), THF (25%) and 5-CHOTHF (20%), whereas the other only initially reported 5-CH_3THF but later confirmed small amounts (10–15%) of THF and 5-CHOTHF forms. Despite the use of HPLC with fluorometric detection, there were some problems in peak identification and calibration. The use of HPLC with UV detection gave unsatisfactory results due to difficulties in resolution of folate compounds and these results were excluded. RPBA results were generally higher (50–60%) than both the microbiological and HPLC results but more variable. EPBA results also varied between the three laboratories using this format but the mean folate content (HP only) agreed favourably with both the HPLC and microbiological results. The major problem identified with these methods was the response of the individual folate forms to the binding-protein used. Careful control of assay pH and calibrant are required if these methods are to be applied to the determination of food folates. Future work will focus on improvements in methodology of each type of assay prior to further intercomparisons.

INTRODUCTION

In 1988, the Commission's Community Bureau of Reference (BCR) undertook a project to improve the quality of vitamin analyses in food, primarily to meet the requirements of the proposed community legislation on the Nutritional Labelling of Foods (EEC Directive 90/946/EEC). The project includes research into improvements in vitamin methodology, intercomparisons of methods between laboratories and preparation of suitably homogeneous and stable reference materials for food use.

The folates represent an important section of the B-group vitamin complex of great nutritional importance because of their role in the synthesis of nucleic acids, and forming one of the anti-oxidant vitamins (Davidson

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et al., 1979). A large number of folate vitamers are known differing in the extent of the reduction of the pteroyl group, the presence of substituents and the number of glutamyl residues attached to the pteroyl group. These vitamers differ in their biological activity and for nutritional purposes it is desirable to measure the individual forms.

At present the most widely used and accepted procedure is the microbiological assay using *Lactobacillus rhamnosus* or *Enterococcus hirae* (formerly known as *Streptococcus faecalis*) as the test organism (Keagy, 1985), where a response of the organism to the mixture of folates present is measured. Even with the recent development of semi-automated procedures including the microtitration plate format (Newman & Tsai, 1986; Horne & Patterson, 1988), 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. In order to measure the polyglutamated forms, preliminary enzymatic deconjugation is essential. The conditions of the deconjugation step need to be optimised especially in the choice of the deconjugase enzyme, pH and length of incubation used. The conditions of the microbiological assay used are critical for precision and in general the procedures are unsatisfactory nutritionally because the different vitamers are not measured individually.

As an alternative, chromatographic procedures using HPLC with either UV or fluorescence detection are available to resolve the forms in standard mixtures but the low levels of folates found in most foods is an added complication. Biospecific methods of analysis based on enzyme-linked immunosorbent assays (ELISAs) and enzyme protein-binding assays (EPBAs) have also been reported for folate (Finglas *et al.*, 1988*a*; Hansen & Holm, 1988) and these appear to offer the necessary specificity and sensitivity for food use.

The results of the first BCR intercomparison of methods for vitamins in food (Hollman *et al.*, in press) concluded that folate was not routinely determined in most of the laboratories taking part. However, due to its nutritional importance, it was agreed to invite specialised laboratories with experience in the analysis of food folates to take part in further work and an intercomparison for the determination of folate was organised.

The overall objectives of the study were to evaluate the performance of the different types of method for folate analysis in food with a view to identifying improved procedures, possibly based on HPLC or biospecific techniques, which potentially offer greater specificity. It was also proposed to examine the performance of current in-house procedures (microbiological assay, HPLC, radioassay and biospecific methods) on distributed samples of lyophilised Brussels sprout. The efficiency of three deconjugase treatments (human plasma, chicken pancreas and hog kidney) would also be investigated. The results of the study are given in this paper.

Table 1. Types of methods used in the first BCR-intercomparison on the determination of folates in food

Method	Number of participants
Microbiological Assay (MA)	8
HPLC	4
Radioprotein-Binding Assay (RPBA)	3
Enzyme Protein-Binding Assay (EPBA)	3

MATERIALS AND METHODS

Participants

An initial questionnaire was sent in October 1990 to those participants who were known to be interested in folate determination in food. In all 15 participants agreed to take part using a range of methods (Table 1). The choice of the method to be used was left to the individual participant subject to the requirement of achieving the best level of accuracy and control of calibration.

Samples

Lyophilised Brussels sprout was prepared as a candidate reference material for vitamin analysis by the Commercial Freeze-drying Company Ltd., Preston, UK in March 1990. It was sent to the AFRC Institute of Food Research, Norwich Laboratory, for milling (≤ 1 mm^2 particle size) and packaging in units of 20 g in food-grade, heat sealed plastic laminate sachets and stored at -30°C. The material had a residual moisture content of 10·5%.

A study of stability and homogeneity was also carried out using a microbiological assay (Phillips & Wright, 1982). Although the long-term stability study of folate in Brussels sprouts has not yet been completed, there is no indication that folate in this material is not stable. Indeed, the short-term stability results indicate that folate is stable in this form for at least 28 d at +25°C (zero time, mean folate = 623 μg per 100 g dry weight, SD = 152; 28 d, 655 μg per 100 g dry weight, SD = 117).

The between-sachet variation was obtained by 20 single determinations of different sachets taken throughout the sampling scheme (mean = 609 μg per 100 g dry weight, SD = 116, % CV = 19). The within-sachet variation was obtained by 10 replicate determinations of the same sachet (mean = 681 μg per 100 g dry weight, SD = 159, % CV = 23).

Deconjugase enzymes

Lyophilised human plasma (HP; Catalogue no. P-9523; 5 ml) was obtained from Sigma, Poole, UK, and prepared by reconstituting the contents of 1 vial of HP with 1 M L-cysteine hydrochloride solution (5 ml). The enzyme solution was stirred gently for 30 min at ambient temperature and then centrifuged at 2500 × g

for 5 min. The supernatant (0·1 ml) was used for sample deconjugation. The HP preparation was prepared fresh on the day of the analysis. Chicken pancreas enzyme (CP) was purchased from Difco, Detroit, Michigan, USA, and prepared by the Laboratory of the Government Chemist, Teddington, UK, according to the method of Bell (1974); 0·5 ml of CP solution was used per sample deconjugation.

Pancreatic enzyme solution

Pancrex V capsules were obtained from Davies & Byrne Ltd., Greenford, Middlesex, UK, and contained the following enzymes: free protease (430 units), lipase (800 units) and amylase (9000 units). The contents of 2 capsules were mixed with 10 ml distilled water for 30 min at ambient temperature. The solution was centrifuged at 2000 × g for 5 min and the supernate used (0·1 ml) for the deconjugation step. The enzyme solution was prepared fresh on the day of analysis.

Pteroylmonoglutamic acid (PGA) calibrant

PGA was obtained from Sigma, Poole, UK (Catalogue no. F7876; ≥ 98% purity) and a 200 μg ml^{-1} solution was prepared as follows. PGA (20 mg) was dissolved in 1 M NaOH solution (0·2 ml), a small volume of distilled water added and the pH adjusted to 6·0 with 1 M HCl. The solution was transferred to a 100 ml volumetric flask with the aid of distilled water, absolute alcohol added (20 ml) and made to volume with distilled water (200 μg ml^{-1}). It was diluted as required for participants using the microbiological assay.

Participants were asked to follow the instructions for preparation and measure the calibrant solution after appropriate dilution. The participants were required to prepare the calibration solution on the day of analysis and store at +4°C in the dark in a refrigerator.

Design of study

The study took place from December 1990 to March 1991. Fifteen participants each received the following items:

(1) Protocol for study including instructions for deconjugation using HP and CP enzymes and preparation of PGA calibrant solution, reporting sheets and method questionnaires;
(2) 1–4 sachets of lyophilised Brussels sprout material;
(3) 1 vial of HP deconjugase enzyme and CP preparation (3 × 2 ml);
(4) Pancrex V capsules (5) and L-cysteine hydrochloride (1 g);
(5) PGA calibrant (20 mg).

Participants were asked to follow the suggested extraction and deconjugation procedures as far as possible (although in the case of the HPLC methods this was not always feasible) and measure the folate content of the samples using their chosen method of end-analysis.

Each participant was required to provide results from at least 5 replicate analyses for each deconjugase treatment and method of analysis, and to give precise details of the methods used on the questionnaires provided.

All participants were asked to provide copies of calibration graphs. In addition, where a HPLC method was included, the participant was asked to supply labelled, representative chromatograms of both the samples and calibrants. Participants were also asked for 4 replicate determinations on the PGA calibration solution after appropriate dilution.

Participants were asked to express the results as follows:

- on a dry weight basis as determined by drying at atmospheric pressure for 4 h at 103 ± 2°C;
- to at least 3 significant figures as μg folate per 100 g dry weight.

Suggested extraction procedure

Lyophilised Brussels sprout powder (1–2 g) was dispersed in 0·1 M phosphate buffer (70 ml; containing 1% ascorbic acid and adjusted to pH 6·0 with 1 M NaOH) in a 250 ml conical flask fitted with an air-condenser. The solution was brought to 100°C on a stirrer/hot plate and gently refluxed for 5 min. The solution was then allowed to cool to room temperature and transferred to a 100 ml volumetric flask, and made to volume with extraction buffer. It was centrifuged at 2000 × g for 10 min and two portions (10 ml) of the supernatant removed and stored in polypropylene tubes at −18°C until required for deconjugation.

HP deconjugation

The deconjugation system consisted of sample extract (3 ml), HP preparation (0·1 ml), pancreatic enzyme solution (0·1 ml) and extraction buffer (6·8 ml). The mixture was incubated at 37°C for 1 h followed by heating at 100°C for 5–10 min in order to inactivate the enzymes. An enzyme blank solution was also included. The solution was transferred to a 25 ml volumetric flask and made to volume with buffer. The flask was stoppered, shaken well and 2 × 5 ml portions of the solution transferred to polypropylene tubes for centrifugation for 10 min at 2500 × g. Duplicate portions of the supernatant were taken and stored at −18°C until required for analysis. These conditions were shown to be optimal for this sample.

CP deconjugation

The deconjugation system consisted of the sample extract (10 ml), ascorbic acid solution (1%, w/v; 1 ml) which has been adjusted to pH 7·0 with 1 M NaOH, and CP enzyme solution (0·5 ml). The solution was mixed using a vortex mixer and incubated for 16 h at

Table 2. Details of the microbiological procedures

Lab. No.	Medium	Organism	Detection	Calibration[a]	Statistical data[b]	Reference(s)
2	Bacto	*Enterococcus hirae* (formerly *Streptococcus faecalis*) (ATCC 8043)	Lactate titration	PGA, 0–15 μg ml^{-1} curve supplied	Reprod. 9·5% (HP) 2·8% (CP)	Bell (1974)
3	Difco (pH 6·2)	*L. rhamnosus* (NCFB 243)	Abs. @ 630 nm using microtitration plate reader	PGA (Sigma), 18 h 0–100 pg per well curve supplied	% CV = 4·7 (HP), 9·3 (CP) % Recovery = 85–115 (PGA)	Phillips & Wright (1982) and Finglas *et al.* (1990)
5	Difco (pH 6·1)	*L. rhamnosus* (ATCC 7469)	Turbimetric @ 595 nm	PGA (Fluka 47620, 97%) 0–1·5 ng per tube curve supplied	In-house RMS: Flour, % CV = 15 (n = 10) Skimmed milk, % CV = 8 (n = 10)	Bell (1974)
6	Difco (pH 6·1)	*L. rhamnosus* (CR[c]) (NCIB 10463)	Turbimetric	PGA (Sigma), 16 h 0–1 ng ml^{-1} curve supplied	Inter-assay CV = 9·7% Intra-assay CV = 2·7% Recovery = 90–100%	Williams (1984)
8	Difco (pH 6·1)	*L. rhamnosus* (ATCC 7469)	Turbimetric @ 575 nm	PGA curve supplied	Repeatability = 25% Recovery = 50–150%	—
10	Difco (pH 6·0)	*L. rhamnosus* (ATCC 7469)	Turbimetric using Corning nephelometer + EEL galvanometer	PGA (Sigma) 0–1 ng per tube curve supplied	% CV = 7·9 (HP), 10·3 (CP)	Bell (1974)
12	DANO (Ferrosan) (pH 6·0)	*L. rhamnosus* (ATCC 7469)	Turbimetric (Abs. @ 650 nm)	PGA 0–1·2 ng per tube	Reprod. 8–16% (peas 14·2) Recovery 95%	Pedersen (1988)
14	Merck (pH 6·2)	*L. rhamnosus* (CR[c]) (NCIB 10463)	Abs. @ 540 nm	PGA (Sigma) 0–1·4 ng per tube curve supplied	Intra- % CV = 5·5 (HP) 6·3 (CP) Inter- % CV = 8·8 (n = 16) in-house QC human serum	Scott *et al.* (1974) & Wilson & Horne (1982)

[a] PGA = pteroylmonoglutamic acid.
[b] HP = Human plasma, CP = Chicken pancreas.
[c] CR = chloramphenicol resistant.

37°C. After incubation, ascorbic acid solution (1 ml) was again added and the solution autoclaved for 15 min at 121°C in order to inactivate the enzyme. After autoclaving, the solution was allowed to cool to room temperature and diluted as required for analysis.

Methods of folate determination

The choice of the method was left to the individual participant subject to the requirement of achieving the best level of accuracy and control of calibration. (Details of the methods used by the participants are given in Tables 2–4).

Treatment of analytical results

The results as received from participants were critically discussed at an evaluation meeting of the participating laboratories in order to detect possible biases and sources of analytical error. In case of any doubt, for example, with regard to poor calibration, the results were rejected on technical grounds. This happened prior to statistical treatment.

The conformity of the distribution of results to a normal distribution was assessed by the Kolmogorov–Smirnov–Lilliefors test. Outlying variances and means were detected by the Cochran and Nalimov tests, respectively. The Bartlett test was used to assess the overall consistency of the variances obtained in the participating laboratories.

RESULTS AND DISCUSSION

General

The majority of the results were received by March 1991. Two participants experienced difficulties with their HPLC methods and only one of these was eventually able to produce some limited results using their HPLC system. One further participant was unable to complete a set of RPBA results for the study. The details of the various methods used by the participants are given in Tables 2–4.

Human plasma deconjugase has several advantages over hog kidney enzyme and was chosen for this study. It is much easier to use and has higher enzymatic

Table 3. Details of the HPLC procedures

	Lab. No. 1	Lab. No. 9	Lab. No. 15
Extraction	2 g + 0·05 M tris-HCl buffer/30% (v/v) methanol containing 5–20 mmol l^{-1} ascorbate. De-gas with N_2. Adjust to 100 ml volume after filtering, centrifuge and store at −20°C. Deconjugation (CP & HP) as per protocol	2 g + 50 ml (0·05 M acetate buffer + 1% ascorbate (pH 4·9), 5 min @ 100°C, centrifuge, add 2 ml hog kidney preparation, incubate 2 h @ 37°C	0·5 g, as given in protocol
Clean-up	—	Anion-exchange column (DEAE-Sephadex A25)	—
Column	Octadecylsilane (RP18) 5 μm 250 × 4·6 mm	(a) Waters μ-Bondapak C18, 10 μm 3·9 mm × 15 cm (b) Waters μ-Bondapak phenyl 10 μm, 3·9 mm × 30 cm pre-column: PAK μ-Bondapak C18	Spherisorb 5 μm, 4·6 mm × 25 cm Phase Separations
Mobile phase	5 mM Tetrabutylammonium—phosphate pH 7·4/30% (v/v) methanol 1 ml min^{-1}	0·033M KH_2PO_4/acetonitrile 90·5:9·5 (v/v), pH 2·3, 1 ml min^{-1}	H_2O/acetonitrile (90 : 10) (v/v), pH 2·3, ml min^{-1}
Detection	280 nm UV	Fluorescence, 292/356 nm (ex/em)	Fluorescence, 296/365 nm (ex/em)
Calibrants	Tetrahydrofolic[1] 5-Formyltetrahydrofolic, 5-Methyltetrahydrofolic and Pteroylmonoglutamic acids	1. Tetrahydrofolic acid, 3HCl. $2H_2O$ 2. N^5-Formyltetrahydrofolic acid (Ca salt) 3. R,S-N^5-Methyltetrahydrofolic acid (Ca salt) One level calibration, 1. 0·1 μg ml^{-1}, 2. 0·05 μg ml^{-1}, 3. 1 μg ml^{-1}	5-methyltetrahydrofolic acid monoglutamate, Schircks, Switzerland 20–100 ng ml^{-1} calibration
RT (min)	THF (9·8), 5-CHOTHF (12·6) 5-CH_3THF (22·0), PGA (28·2)	1. 8·50–9·00, 2, 10·50–11·00 3. 20·00–22·00	*Std.* 14·0–14·4 & 10–12, *HP* : 11·6 *CP* : 10·8
Statistical Data:	—	Reprod. ±10%, Repeat. ±5%, Recovery 90%	%CV = 14·2 (HP), 10·2 (CP) and 11·1 (no enzyme)
Reference	Shulz *et al.*, (in press)	Gregory *et al.*, (1984)	Curtius *et al.* (1989)

Table 4. Details of the radioprotein-binding (RPBA) and enzyme protein-binding (EPBA) procedures

Lab No.	Type	Principle	Extraction	Calibrant	Detection	Statistical date	Reference
4	RPBA[a]	Radiobinding assay using-milk-binding protein and ^{125}I-PGA as tracer	As per protocol. Mix 0·2 ml sp. or std. with tracer, incubate with denaturation agent + KCN at pH 12·3–13·0	PGA (0–20 ng ml^{-1}) supplied in kit. Curve supplied	Scintillation counting	Serum (n = 20). Within-assay CV = 5·1%. Between-assay CV = 10·9% Recovery = 87–106%	—
13	As for (4)	As for (4)	As per protocol using 1·0 g sp.	As for (4). No curve supplied	As for (4)	As for (4)	—
3	EPBA	Competition between free folate added to well and bound folate on surface of well for binding sites on an enzyme-labelled milk binding protein	As per protocol. 1 ml sp. add 5 ml PBST + 1% Ascorbate (pH 7·4), check pH and adjust if necessary to pH 7·4 with 1N NaOH. Make to 10 ml volume with buffer. Dilute in buffer ×25 and ×50 for assay	5-MTHFA (Schircks) Stock soln.: 10 μg ml^{-1} in PBST + 1% Ascorbate (pH 7·4) based on extinction coefficient (abs. @ 290 nm = 0·65 a.u.). Store stock at −20°C in small aliquots. Dilute to give 10, 25, 50, 100, 250, 500 & 1000 pg per well	Microtitration plate reader @ 450 nm after stopping reaction with 50 μl 2N H_2SO_4	Stds. % CV = 5·0 sps. % CV = 5–10·0	Finglas *et al.* (1988*a*)
7	EPBA	Based on Lab(3) with the following modifications: FBP linked to *N*-hydroxy-succinimido-biotin; avidin-alkaline phosphatase was used as enzyme conjugate. Plates coated with FA-KLH	As suggested in protocol using 5·0 g sample weight	PGA (98% pure, Sigma) 5-MTHFA (Na Salt, 90–95%, Sigma) Curves supplied for (1) PGA, (2) 5-MTHFA & (3) HP treated 5-MTHFA. Sp. calculated from (2) & (3), PGA calibrant from (1)	Microtitration plate reader	Repeatability HP: 5MTHF 211·5 × 378 CP : FA 11·28 × 65 (Expressed as 95% critical difference interval). Recovery FA 78% 5-MTHF 75%	—
12	EPBA	Competitive indirect EPBA based on BSA-folate on plates, FBP-biotinylated & avidin-alkaline phosphatase conjugate	1 g (as MA)	D,L-5-methyltetrahydrofolic acid, Ba-salt (Sigma) (ref. *Clin. Chem.* **33**, (1987) 1360–3	Microtitration plate reader or video image processing	Repeatability— %CV < 10·0 for PGA (1·5–150 nmol)	Hansen & Holm (1988)

[a] Commercial kit.

Table 5. Folate content (μg per 100 g dry weight) of Brussels sprout by microbiological assay using human plasma deconjugation

Lab No.	Folate content[a]					Mean	SD	% CV
	1	2	3	4	5			
2[b]	94[a]	107[b]	104[c]	102[d]	—	101·8[b]	5·6	5·5
3	734	768	798	718	—	754·5	35·7	4·7
5	1070	1011	1046	1001	—	1032·0	31·8	3·1
6	736	646	688	689	681	688·0	32·0	4·7
8[c]	417	389	397	389	—	398·0[c]	13·0	3·3
10	748	809	729	664	683	726·6	57·0	7·8
12	1047[a]	1044[b]	996[b]	936[d]	916[d]	987·8	60·4	6·1
14	825	733	721	757	738	754·8	41·0	5·5
					Overall mean (n = 6)	824·0	146·8	17·8

[a] Replicate determinations of the same sachet unless otherwise indicated by superscript letter.
[b] Results from Lab. 2 excluded in overall mean due to use of *E. hirae*.
[c] Results from Lab. 8 excluded in overall mean due to inadequate calibration.

Table 6. Folate content (μg per 100 g dry weight) of Brussels sprout by microbiological assay using chicken pancreas deconjugation

Lab No.	Folate content[a]					Mean	SD	% CV
	1	2	3	4	5			
2[b]	282[a]	286[b]	278[c]	277[d]	—	280·8[b]	4·1	1·5
3	1450	1476	1554	1460	1201	1428·2	133·0	9·3
5	1045	1054	—	—	—	1049·5	—	—
6	793	763	770	750	810	777·2	24·0	3·1
8[b]	1120	480	453	640	267	592·0[b]	320·0	54·1
10	1086[a]	914[a]	854[a]	864[d]	985[b]	940·6	96·0	10·3
12	853[a]	846[b]	842[c]	840[d]	812[d]	838·6	15·7	1·9
14	964	864	836	857	828	869·8	55·0	6·3
					Overall mean (n = 6)	984·0	236·8	24·1

[a] Replicate determinations of the same sachet unless otherwise indicated by superscript letter.
[b] Results from Lab. No. 2 excluded in overall mean due to use of *E. hirae* and Lab. No. 8 due to large variation.

activity, thus permitting the use of shorter incubation times and reducing the risk of potential folate degradation. It can also be used at a pH of 6·0 compared to about pH 4·6 for hog kidney, this being useful as folates tend to be more stable under alkaline conditions. Marked losses of folates, notably 10-formyltetrahydrofolic and tetrahydrofolic acids, have been reported during incubation at pH of about 4·6 (Butterfield & Calloway, 1972; McMartin *et al.*, 1981).

The pancreatic enzyme preparation was also included in the extraction system because it contains an α-amylase. Other workers have found that significant increases in measurable folate determined by microbiological assay, can be observed in a number of foods if the α-amylase is used in addition to the deconjugase enzyme (Pedersen, 1988; De Souza & Eitenmiller, 1990).

Microbiological procedures

The microbiological results for HP and CP deconjugase enzymes are given in Tables 5 and 6 and Figs 1 and 2, respectively. Although there was a tendency for the CP treated samples to give higher folate levels in this sample, this was not found to be statistically significant ($P > 0·05$; paired t-test). Folate levels from the CP treated samples were 13–89% higher compared to those samples treated with HP deconjugase in five laboratories with acceptable microbiological results. The remaining laboratory found higher (~15%) folate levels in the HP samples.

Laboratory 2 used *E. hirae* (formerly *Streptococcus faecalis*) and as this organism does not respond to the methyl-folate forms, results from this laboratory were omitted in the calculation of the overall mean. (The HPLC data indicated that 47–68% of the folate present in the Brussels sprout material was in this form.) If the folate results using CP from laboratory 2 are multiplied by a factor of 2, assuming that 50% of the folate is in the methyl form and is not measured using *E. hirae*, then the results fall on the lower range of values reported by the other laboratories. The HP results from this laboratory are still on the low side compared to the other laboratories but this laboratory reported difficulties with the HP blanks. The CP preparation was purified

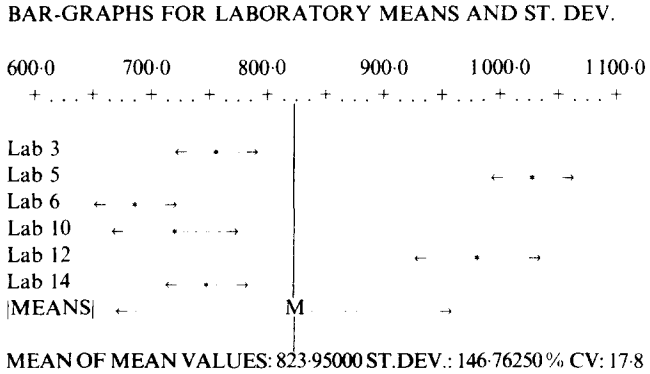

Fig. 1. Bar-charts (mean ± 1 SD) of accepted microbiological results (μg per 100 g dry matter) of total folate in Brussels sprout using human plasma (HP) deconjugase enzyme.

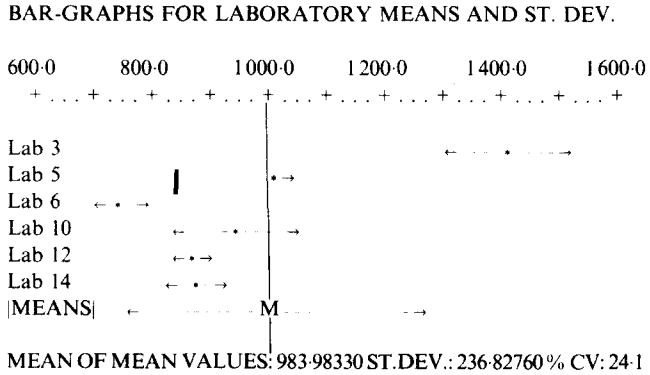

Fig. 2. Bar-charts (mean ± 1 SD) of accepted microbiological results (μg per 100 g dry matter) of folate in Brussels sprout using chicken pancreas (CP) deconjugase enzyme.

Table 7. Microbiological results (μg ml⁻¹) of PGA calibrant[a] (theoretical value = 200 μg ml⁻¹)

Lab No.	PGA reported[b]					Mean	SD	% CV
	1	2	3	4	5			
3	218	233	210	228	223	222·4	8·9	4·0
5	212	204	212	214	—	210·0	4·0	1·9
6	189	193	189	187	196	191·0	3·6	1·9
10	220	240	230	245	—	233·8	11·0	4·7
12	171·6 182·3	182·2	188·8	172·6	185·7	180·5	7·0	3·9
14	200	213	206	200	213	206·4	6·3	3·1
			Overall mean (n = 6)			207·4	19·6	9·5

[a] PGA = pteroylmonoglutamic acid.
[b] Replicate determinations of the same calibration solution.

to some extent and therefore enzyme blanks did not appear to be such a problem.

The results from laboratory 8 were also questionable since this laboratory reported problems with the reproducibility/repeatability of their microbiological assay. Thus, the folate results using CP from laboratory 8 were omitted from the overall mean value as a result of unacceptably high variation in the calibration curve and poor sample duplication. However, their results using HP are better, despite being lower than the other laboratories using microbiological assays but have also been omitted for the general problems of calibration.

In general, calibration did not appear to be a problem in the microbiological assay from the results of the PGA calibrant supplied (Table 7). Good agreement was found between laboratories (% CV = 9·5) and the overall mean of 207·4 μg ml⁻¹ compares favourably with the theoretical value of 200 μg ml⁻¹.

The effect of different extraction conditions (time, temperature) on folate levels determined by microbiological assay was also investigated by one laboratory. The results are shown in Fig. 3. The use of autoclaving at 121°C for 15 min followed by deconjugation with

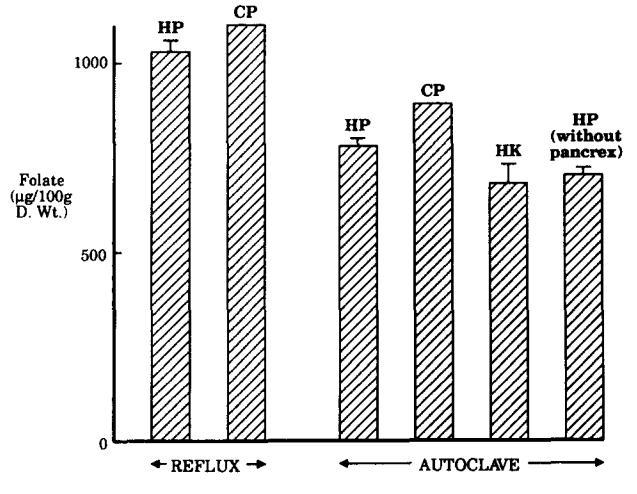

Fig. 3. Effect of extraction conditions (reflux and autoclave) and deconjugase enzyme (human plasma, HP; chicken pancreas, CP and hog kidney, HK), on folate levels (μg per 100 g dry matter) as determined by microbiological assay.

either HP or CP gave 24% and 11% lower folate values respectively compared to the same enzyme and refluxing at > 90°C for 5 min. The microbiological conditions in the Bell (1974) procedure include two autoclave treatments, the second to deactivate any remaining enzymes. Even with the addition of an antioxidant, for example ascorbic acid, prior to each autoclaving, significant folate losses can still occur.

A third deconjugase enzyme, hog kidney (HK) was also used with autoclaving and this gave lower folate levels (14% and 28%) compared to HP or CP enzymes with autoclaving. HK would therefore appear not to be as effective as HP or CP deconjugase enzymes in this particular food possibly due to the presence of enzyme inhibitors found in brassicas. The HP treatment without the pancreatic enzyme and autoclaving was not as effective as HP or CP with pancreatic enzyme and either autoclaving or refluxing.

One participant reported that samples treated with CP showed a similar growth response to the PGA calibrant. Samples treated with HP or HK, however, both showed positive deviation from the standard growth response resulting in significant 'drift' of results assayed at different concentration ranges. Folate values of 0–50 μg per 100 g (20%) in the enzyme blanks, notably the pancreatic enzyme, were also found by two participants.

Other laboratories reported very low folate levels in the enzyme blank samples. The use of an overnight incubation for deconjugation (as suggested by Bell, 1974) can increase the risk of growth of non-folate requiring bacteria which leads to competition with the assay inoculum and hence an overestimate of the folate levels in these samples. In this study participants were asked to incubate overnight for the CP treatment but only for 1 h for HP, as this was found to be optimal for this type of sample.

HPLC procedures

The HPLC results for the two participants who were able to complete the study are given in Table 8 and Fig. 4. Laboratory 9 reported THF, 5-CH_3THF and 5-CHOTHF whereas laboratory 15 only initially found the methyl form but further work identified low levels of THF (15–20% of the 5-CH_3THF found). The results from laboratory 9 indicate that folate levels are similar for both HP or CP enzymes but HK values were about 20% higher. It is unclear why this laboratory found no differences in folate levels with or without deconjugation especially as laboratory 15 reported increases of 87% and 115% in folate levels after treatment with HP or CP deconjugase enzymes, respectively. Similar extraction conditions were used by both laboratories.

Total folate values in Brussels sprout by HPLC given by laboratory 9 agree well with the microbiological results (Fig. 5). Laboratory 15 only initially reported 5-CH_3THF and values were about 15% higher than the corresponding data given by laboratory 9 for this folate form. There would appear, therefore, to be a problem

Table 8. HPLC results of (μg per 100 g dry matter) folate in Brussels sprout expressed as individual and total folate using human plasma (HP), chicken pancreas (CP) and hog kidney (HK) deconjugase enzymes

Lab. No.	Extraction conditions	Enzyme	THF (% total)	5-CH_3THF (% total)	5-CHOTHF (% total)	Total
9[a]	5 min/≥90°C	HP	160 (21)	354 (47)	248 (33)	762
	5 min/≥90°C	CP	194 (27)	390 (54)	145 (20)	729
	5 min/≥90°C	HK	210 (23)	576 (62)	143 (15)	929
	30 min/≥90°C	HK	168 (20)	564 (68)	97 (12)	828
	5 min/≥90°C	No enzyme	211 (25)	504 (59)	145 (17)	859
	15 min/≥90°C	No enzyme	276 (32)	476 (55)	111 (13)	863
15[b]	5 min/≥90°C	HP	nd	877 760 997 787 917 Mean = 868 SD = 96·5 % CV = 11·1	nd	—
	5 min/≥90°C	CP	nd	915 1115 1055 876 945 Mean = 981 SD = 100·2 %CV = 10·2	nd	—
	5 min/≥90°C	No enzyme	nd	439 376 463 488 555 Mean = 464 SD = 65·6 % CV = 44·1	nd	—

NB: nd = not determined.

[a] Single analysis of 1 sachet, 5–10 injections.

[b] Two readings on five separately weighed samples. THF and 5-CHOTHF were not determined quantitatively, although small amounts were detected (see Fig. 8).

in either calibration or peak identification in one or other methods.

Both laboratories 9 and 15 used reverse phase systems and fluorometric detection (see Table 4). The native fluorescence of THF, 5-CH_3THF and 5-CHOTHF can be adequate to permit direct detection and quantification. Chromatograms of standards and samples with and without deconjugation are shown in Figs 6 and 7 respectively. Even with this type of detection there are numerous unidentified peaks which complicate identification and quantification of individual folate forms.

One of the major problems in the use of HPLC for the determination of folates in food is the specificity of

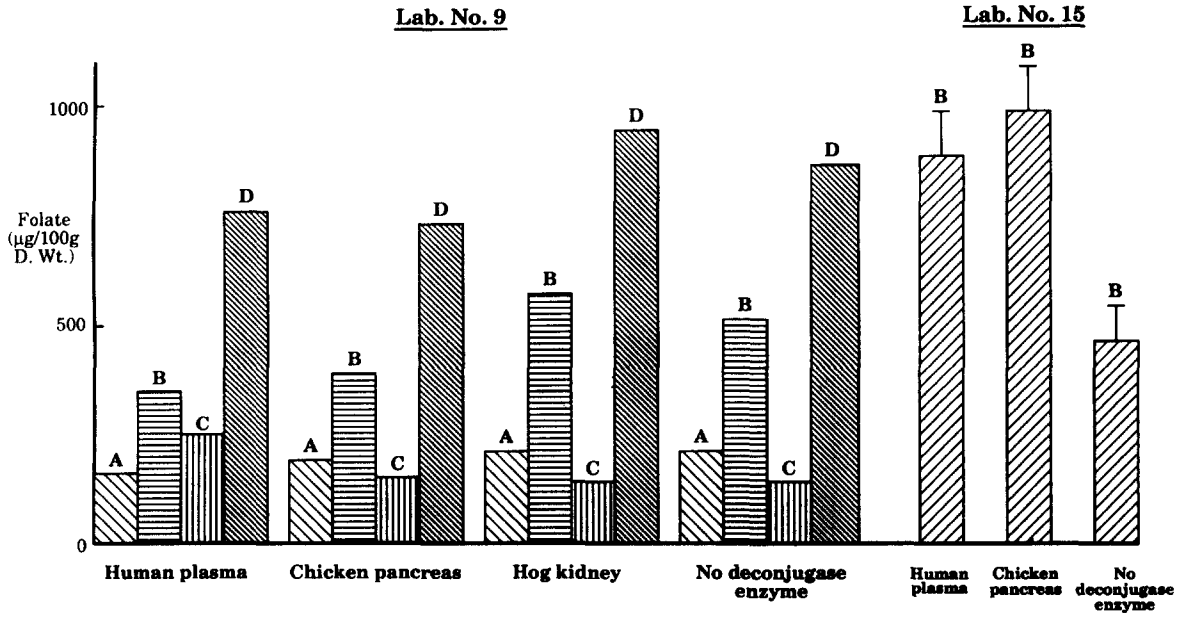

Fig. 4. Distribution of folates (μg per 100 g dry matter) in Brussels sprout by HPLC. (A) tetrahydrofolic acid, (B) 5-methyltetrahydrofolic acid, (C) 5-formyltetrahydrofolic acid and (D) total folate ((A) + (B) + (C)). For details of methods, see Table 3, participants 9 and 15.

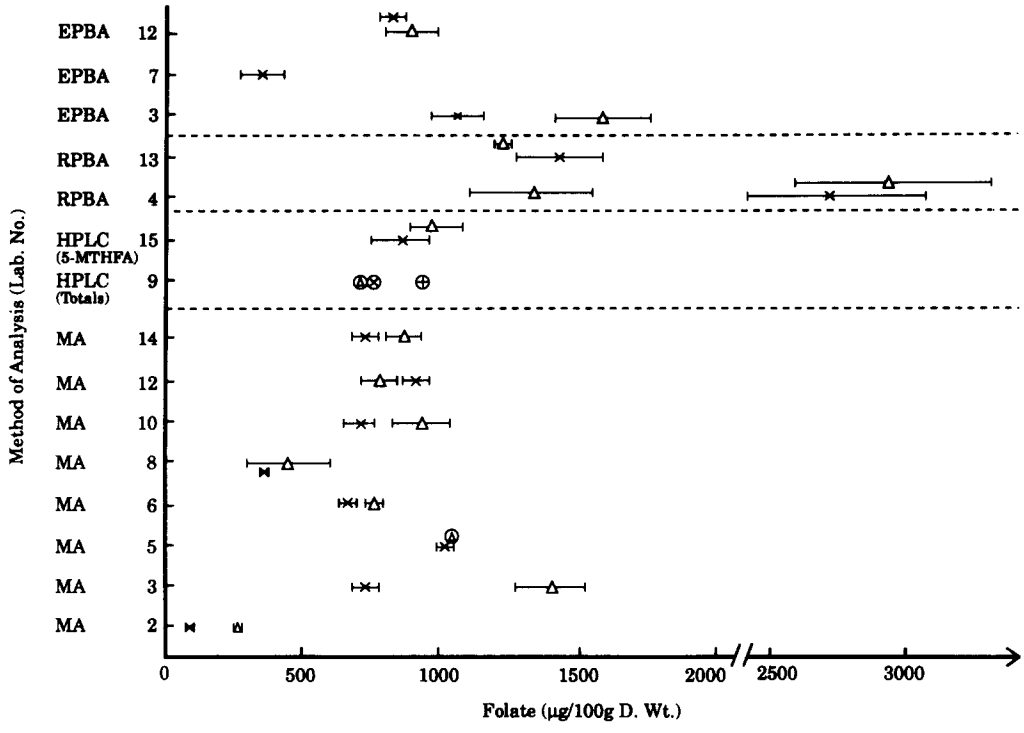

Fig. 5. Comparison of folate levels (μg per 100 g dry matter) in Brussels sprout by a range of techniques. EPBA, protein-binding assay; RPBA, radioassay; HPLC, high performance liquid chromatography and MA, microbiological assay. (Δ) chicken pancreas; (×) human plasma and (+) hog kidney deconjugase enzymes. Results are means ± 1 SD.

the detection of individual forms. This can be especially true if CP enzyme is used for the deconjugation step as a mixture of folate diglutamates will be produced from the use of this enzyme, together with any monoglutamate forms present initially. A further complication is that folate calibrants are only available for PGA in the diglutamate form and not for the naturally occurring folates. Peak identification is commonly made by reference to the retention time of the known material and by spiking with a solution of that material. If a calibrant for a particular folate compound is not available and this form is present in the food extract, then the sum of the individual folate forms determined by HPLC will differ from values obtained by the microbiological assay, for instance, where the test organism should respond equally to all the folate forms.

The determination of food folates for nutritional purposes requires both the total folate level and levels of the individual forms present in the food. The use of HPLC is currently the only viable alternative to the MA if information on the amounts and types of the individual folate forms is required. The most recent development of biospecific methods, notably the availability of antibodies specific to individual folate forms, is an area of much future work. Antibodies could be used either to assist the HPLC procedure in establishing peak identity, or by direct quantitation of the folate form by means of its use in an ELISA system.

The identification of peaks in the HPLC procedure can only be made unequivocally by the use of mass spectrometry (MS). A LC–MS technique for the determination of food folates has been developed by one of the participants in this study (Schulz *et al.*, in press) but due to technical difficulties they were unable to use this procedure in the present study.

The third participant who submitted results for the study used a LC system with UV detection. Although reasonable resolution of folate calibrants was obtained (Fig. 8(A)) using his form of detection, the resolution for samples with both deconjugase enzymes (Fig. 8(B) and (C)) was unsatisfactory and these results were excluded. Only the 5-CH_3THF form was partially resolved in the samples but this participant reported considerably higher folate values compared to the other laboratories using HPLC methods. This could be due partly to the different extraction procedure at a more basic pH used by this laboratory, or improper calibration.

RPBA AND EPBA

Several reports in the literature can be found of the analysis of folate in food using radioassay procedures (RPBA), and their comparison to other folate procedures, notably the microbiological assay. Results from the MA and the RPBA are frequently contradictory (Gregory, 1985).

A number of factors are known to affect the performance of the RPBA. In particular, the folate binding affinity of the binding protein can be influenced by temperature, incubation time, and dramatically by pH. The unlabelled folate calibrant selected for the RPBA must be stable and have an affinity for the binding protein that is comparable to that of the folate form or forms present in the samples. PGA, which is more

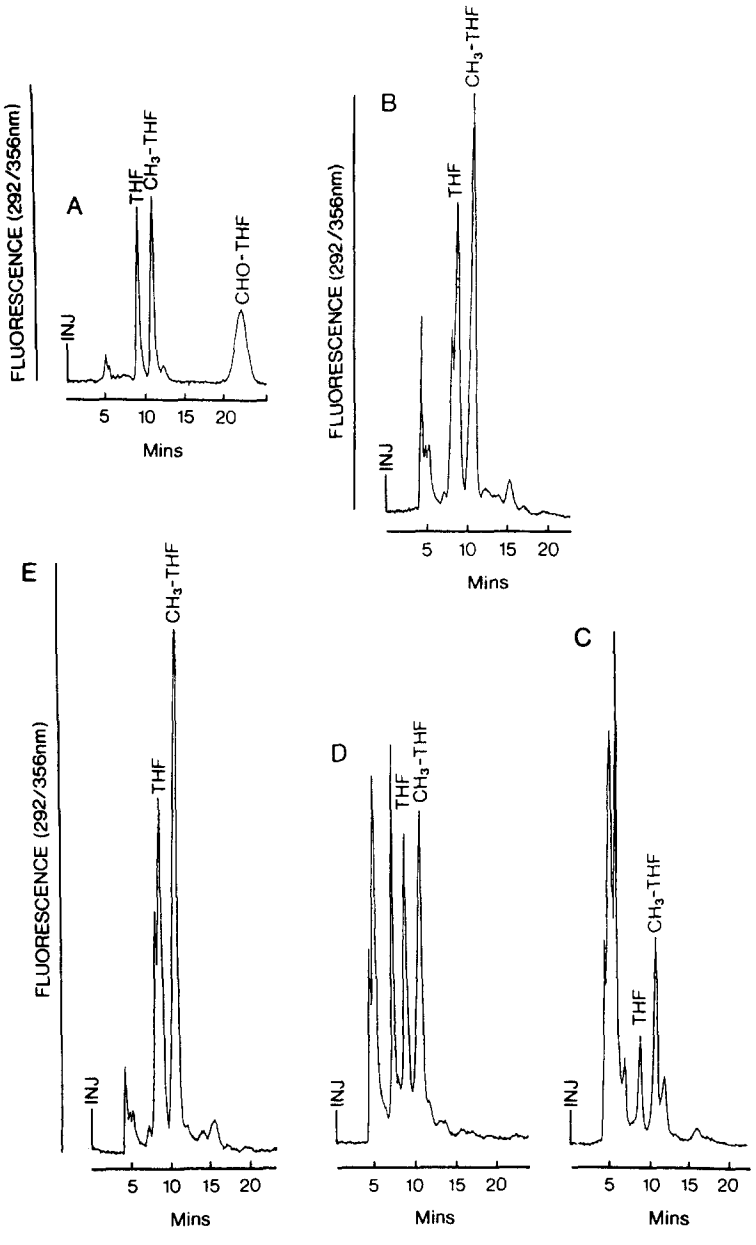

Fig. 6. Chromatograms using fluorometric detection (ex, 292 nm; em, 356 nm) of (A) folate standards, (B) sample extracts after no deconjugation, (C) sample extracts after HP deconjugation, (D) sample extracts after CP deconjugation, and (E) hog kidney deconjugation. For conditions, see Table 3, participant 9.

stable than 5-MTHF, should not be used as the assay standard in assays run at pH 7·2–8·0, because of its greater affinity for the folate binding protein (FBP) compared to that of 5-CH_3THF at this pH (Givas & Gutcho, 1975). However, at pH 9·3, PGA is preferred as the assay standard because of its greater stability and equivalent binding affinity compared to the 5-CH_3THF form.

Several commercial RPBA kits have also been examined for their responses to various folate forms and the results compared to the MA. Although careful standardisation can justify the use of the RPBA method for the analysis of 5-CH_3THF in blood, the variable response of the RPBA kit to different folate vitamers indicates that the application of these methods to food systems may yield tenuous results. To date, their application to foods is limited and not conclusive.

In the present study, two participants used commercial RPBA kits (both from Amersham) and three participants EPBA methods. The results are given in Tables 9 and 10. The RPBA results from laboratory 4 are 90–136% higher compared to laboratory 13 for HP and CP deconjugase enzymes. This is despite the fact that both laboratories used the identical type of RPBA kit from Amersham. It should be noted, however, that this kit has been specifically designed for the analysis of folate in blood, i.e. the determination of the 5-CH_3THF form, and its validation for use in the food area has not been reported.

Laboratory 4 reported that the samples were initially diluted with distilled water and not with the kit diluent provided which is a zero standard with a composition similar to that of human plasma. This laboratory indicated that their results may therefore have been overestimated by about 30–40%. The analysis of the

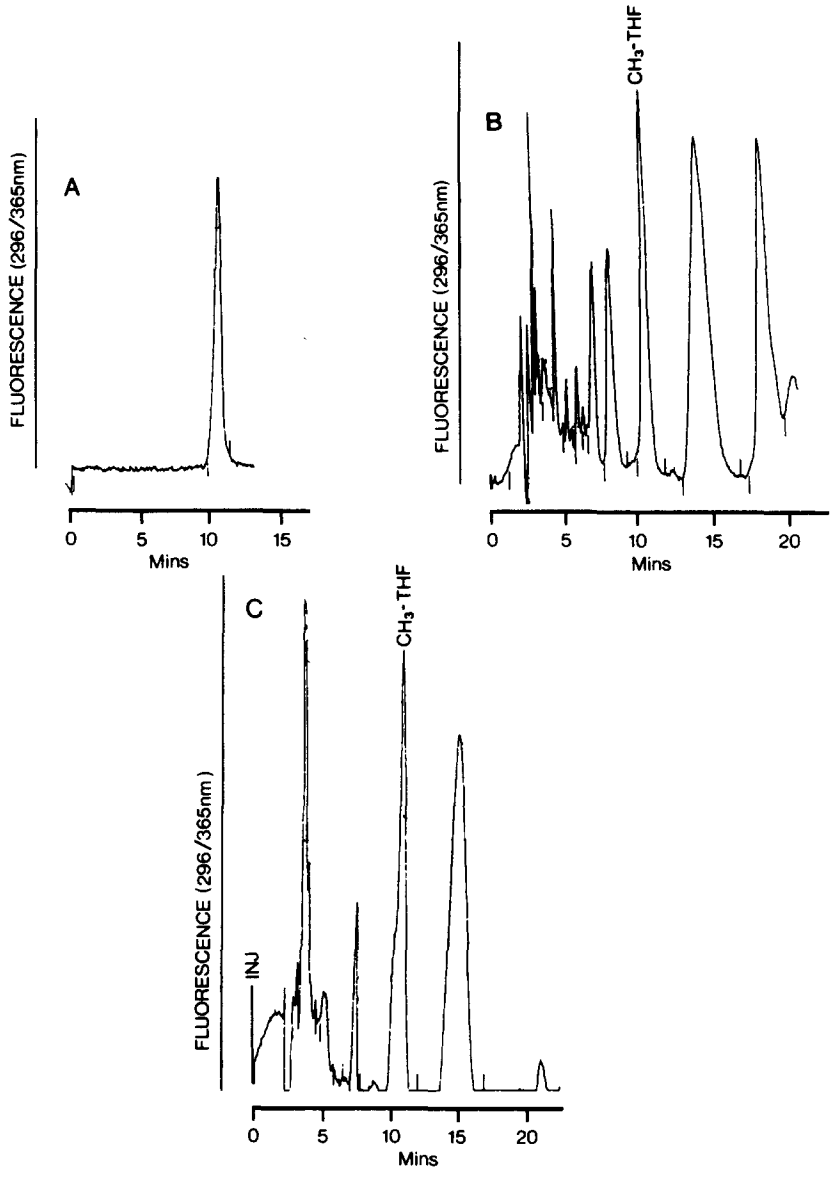

Fig. 7. Chromatograms using fluorometric detection (ex, 296 nm; em, 365 nm) of (A) 5-methyltetrahydrofolic acid standard, (B) sample extracts after HP deconjugation, and (C) sample extracts after CP deconjugation. For conditions, see Table 3, participant 15.

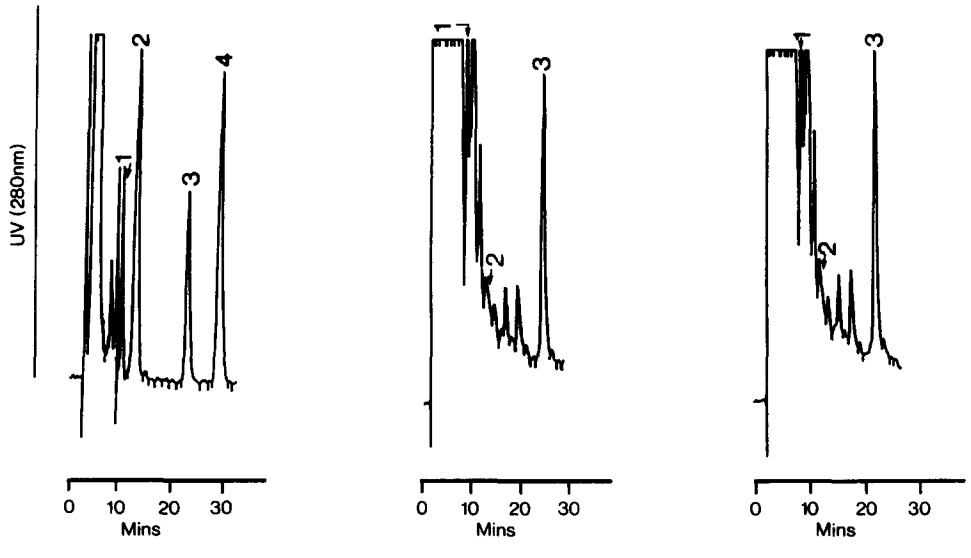

Fig. 8. Chromatograms using UV detection at 280 nm of (A) folate standards, (B) samples after HP deconjugation, and (C) samples after CP deconjugation. (1) THF, (2) 5-CHOTHF, (3) 5-CH_3THF, and (4) PGA. For conditions, see Table 3, participant 1.

Table 9. Folate content (μg per 100 g dry matter) of Brussels sprout by radioprotein-binding (RPBA) and enzyme protein-binding (EPBA) assays using human plasma (HP) and chicken pancreas (CP) deconjugase enzymes

Lab. No.	Assay	Enzyme	Folate (μg per 100 g dry matter)[a]					Mean	SD	% CV
			1	2	3	4	5			
4	RPBA	HP	2190	2920	2940	2630	3020	2740	341	12·5
		CP1[b]	2780	2920	3480	2670	—	2960	360	12·2
		CP2[b]	1869	1048	1191	1193	1324	1325	319	24·1
13	RPBA	HP	1683	1433	1410	1433	1264	1445	151	10·4
		CP	1237	1252	1252	1237	1296	1255	24	1·9
3	EPBA	HP	988	1140	1026	—	—	1051	79	7·5
		CP	1665	1672	1434	1419	1814	1601	170	10·6
7	EPBA	HP	342	467	450	330	300	378	75	19·8
12	EPBA	HP	831	815	805	576[c]	918	842	52	6·2
		CP	1580[c]	868	933	812	999	903	81	9·0

[a] Replicate determinations of the same sachet.
[b] CP1, initial analysis *without* the kit diluent.
CP2, repeat analysis *with* the kit diluent.
See text for details.
[c] Rejected as an outlier.

PGA calibrant using the RPBA by laboratory 4 gave a value of 264 μg ml^{-1}, which is 32% higher than the theoretical value of 200 μg ml^{-1} (Table 10). Even taking this level of overestimation into account, the RPBA results for the Brussels sprout samples from laboratory 4 would still be about 30% and 60% higher for HP and CP enzymes respectively compared to the RPBA results from laboratory 13, and also considerably higher than the majority of the MA and HPLC results.

Laboratory 4 repeated the analyses on the CP treated samples using the RPBA and the kit diluent provided and the mean result was much lower, 1325 μg per 100 g dry matter. This compares favourably with the RPBA result from laboratory 13 of 1255 μg per 100 g for these samples. Other workers have reported that the RPBA cannot be relied upon for the quantification of complex mixtures of folate unless something is already known about the type and distribution of the folate form in the material (Gregory, 1985). The results from this study would support these findings.

The results for the three EPBAs are also given in Tables 9 and 10. Laboratory 7 reported their assay was based on a modification of the published EPBA developed by laboratory 3 (Finglas *et al.*, 1988*a*). Although both assay systems are based on the same folate binding protein from milk, the formats of the assay are different. The EPBA used by laboratory 3 has been previously used for the determination of folate in vegetables, and in this instance good agreement was found between the results from it and a microbiological assay (Finglas *et al.*, 1988*b*). The folate form used for calibration was 5-CH_3THF and its binding affinity was similar to that for 5-CHOTHF and THF using this particular assay system, although somewhat different to that for PGA (Finglas *et al.*, 1988*a*). It was found to be necessary to add ascorbate solution (1%, w/v) to the

Table 10. Radioprotein-binding (RPBA) and enzyme protein-binding (EPBA) assay results (μg ml^{-1}) of folate calibrants (theoretical value = 200 μg ml^{-1})

Lab. No.	Assay	Calibrant[a]	Folate[b]					Mean	SD	% CV
			1	2	3	4	5			
3	EPBA	MTHF	228	236	228	256	248	239·2	12·5	5·2
4	RPBA	PGA	280	240	280	280	240	264·0	22·0	8·3
4	RPBA	PGA[c]	186[c]	176[c]				181·0		
7	EPBA	PGA	193	187	185	197	191	191·0	4·8	2·5
12	RPBA	PGA	210	236	246	218	—	228	16·4	7·2
13	RPBA	PGA	218	200	220	212	230	216	11·1	5·1
	RPBA	MTHF						228		

[a] MTHF = 5-methyltetrahydrofolic acid.
PGA = pteroylmonoglutamic acid.
[b] Replicate determinations of the same calibration solution.
[c] Repeat determinations using kit diluent (see text for details).

Table 11. Comparison of folate levels (μg per 100 g dry matter) of Brussels sprout by different procedures and deconjugase enzymes

Procedure[a]	Deconjugase enzyme[b]	Mean folate (no. of data sets)
MA	HP	824 (6)
	CP	984 (6)
EPBA	HP	757 (3)
	CP	1320 (2)
RPBA	HP	1445 (1)
	CP	1290 (1)
HPLC (total)	HP	762 (1)
	CP	729 (1)
	HK	929 (1)
	No enzyme	859 (1)
HPLC (5-CH_3THF)	HP	868 (1)
	CP	981 (1)
	No enzyme	464 (1)

[a] MA = microbiological assay; EPBA = enzyme protein binding assay; RPBA = radioprotein binding assay; 5-CH_3THF = 5-methyltetrahydrofolic acid.
[b] HP = human plasma; CP = chicken pancreas and HK = hog kidney deconjugase enzymes.

assay buffer used (phosphate buffered saline, pH 7·4) and readjust the pH to 7·4, to protect the 5-CH_3THF calibrant from any risk of oxidative loss in solution during the incubation steps in the assay. Hence, the application of this particular EPBA system for the analysis of folate in Brussels sprout can be considered justified on the grounds that these folate forms appear to be the most predominant forms present.

The EPBA results from laboratory 3 for CP treated samples are in reasonable agreement with the MA data from the same laboratory, although about 25% higher in the case of the HP samples. Their results are also higher than those from laboratories 7 and 12. The calibration ranges used by laboratories 3 and 7 are also considerably different, reflecting the differences in assay format. Laboratory 3 reports an assay calibration range of 0·1–1·0 ng 5-CH_3THF ml^{-1}, whereas laboratory 7 reports 50–100 ng ml^{-1}. This is rather surprising as laboratory 7 incorporates an avidin–biotin amplification system in this assay which should lead to more sensitive detection limits.

The EPBA results from laboratory 12 are comparable to the results obtained by the microbiological assays but 20% and 44% lower than the EPBA data from laboratory 3. The specificity of their assay suggests that PGA and 5-CH_3THF bind equally well but the binding of THF and 5-CHOTHF is much less (Hansen & Holm, 1988). It is also important to stabilise this folate form in solution by use of ascorbic acid, for instance, because of the stability problems of this form in solution.

In this study deconjugation was employed prior to determination by the RPBA and EPBA. Some investigators have reported that the RPBA determinations of folate in food using the milk-binding protein measure folates regardless of pteroylglutamate chain length (Tigner & Roe, 1979) whereas other workers report that the deconjugase treatment may make more folate available for measurement by the RPBA technique in certain samples (Klein & Kuo, 1981; De Souza & Eitenmiller, 1990). From the limited data available on the folate levels in Brussels sprout determined by RPBA and EPBA, there does not appear to be any difference in the use of HP or CP as the deconjugase treatment. However, comparison of RPBA and EPBA data with both the microbiological and HPLC results, suggests that a greater amount of folate-like substances is being measured using the RPBA and EPBA methods (Table 11, Fig. 5).

CONCLUSIONS

This study has indicated that reasonable agreement between laboratories for the determination of folates in food can be obtained by use of the microbiological assay. Further improvements should be possible by the optimization of the extraction and deconjugation steps especially the choice and preparation of the deconjugase enzyme.

The use of HPLC and RPBA/EPBA techniques are still very much in the developmental stage and additional work is needed before the results from these assays can be confidently compared to those of the microbiological assay. However, specific and more rapid procedures which allow the identification and quantification of individual folate forms are needed for nutritional studies.

Greater attention should be given to the identification of unknown peaks in the HPLC separation especially the application of LC–MS systems, and calibration of the various folate forms. The use of sample clean-up should also be investigated and a deconjugase enzyme which only gives folate monoglutamates on deconjugation should be used for HPLC work in order to simplify the number of peaks in the chromatographic separation.

The major limitation of both the RPBA and EPBA would appear to be the response of the individual folate forms to the folate binding protein used. Careful control of the pH of the assay buffer used and choice of the folate calibrant is required if these assays are to be applied to the determination of food folates.

Future work will focus on improvements in methodology of each type of assay prior to a second intercomparison of methods on a wider range of materials.

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