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Standardisation of HPLC techniques for the determination of naturally-occurring folates in food

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Abstract

The aim of this work was to evaluate current in-house HPLC procedures for the determination of naturally-occurring folates in food, and to identify problem areas for further improvement. Five intercomparison studies were completed over the period 1990–1997 in which nine participants from six countries took part. Through careful validations and detailed discussions held at evaluation meetings, possible biases and sources of systematic error have been identified and reduced. The use of ascorbic acid and nitrogen flushing during extraction, sample clean-up using strong anion exchange columns, spectrophometrically calibrated standards and fluorescence detection are all recommended. Both in-house hog kidney and human plasma deconjugase enzymes gave similar results to the circulated common hog kidney enzyme which was prepared from fresh pig's kidneys. The most consistently reported values were for 5-CH_3H_4-PteGlu, and to a lesser extent, for H_4PteGlu. Four candidate reference materials (CRM 121, wholemeal flour; CRM 421, milk powder; CRM 485, lyophilised mixed vegetables, and CRM 487, lyophilised pig's liver) have been proposed with both indicative values (mean ± uncertainty) for 5-CH_3H_4-PteGlu in CRM 421 (0.25; ± 0.02 mg/kg) and CRM 485 (2.14; ±0.42 mg/kg), and information values (mean; range) for 5-CH_3H_4-PteGlu in CRM 121 (0.04; 0.03–0.08 mg/kg) and CRM 487 (2.6; 1.9–3.8 mg/kg). Certified values are also given for total folate by microbiological assay: CRM 121 (0.50; ±0.07 mg/kg), CRM 421 (1.42; ±0.14 mg/kg), CRM 485 (3.15; 0.28 mg/kg), and CRM 487 (13.4; 1.3 mg/kg). Average recovery of 5-CH_3H_4-PteGlu, added prior to extraction and deconjugation, was 91% (84–95%) for the four CRMs. The average within- and between-laboratory variations were 6 and 15% for the determination of 5-CH_3H_4-PteGlu by HPLC, and 9 and 18% for the determination of total folate by microbiological assay. These CRMs will be used for quality control of folate measurements for nutritional labelling, and validation of new techniques. Further methodology work is required for the HPLC analyses of folate forms other than 5-CH_3H_4-PteGlu.

Keywords: Folates; Food; HPLC; Intercomparison; Certified reference materials

1. Introduction

Folates represent a B-group vitamin of great nutritional importance. They are involved in one-carbon transfer reactions required in many metabolic pathways, including purine and pyrimidine biosynthesies, as well as amino acid interconversions (Krumdieck, 1990). There is renewed interest in folate absorption and metabolism due to findings that dietary folic acid supplementation can significantly reduce the risk of neural tube defects in babies (Wald, 1991). In addition, a growing body of evidence is suggestive of a positive association between folate intake and a reduction in the risk of occlusive vascular disease (Boushey, Beresford, Omenn, & Motulsky, 1996; Morrison, Schaubel, Desmeules, & Wigle, 1995).

The vitamin exists in nature primarily as reduced one-carbon substituted forms of pteroylglutamates, differing

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Abbreviations: PGA, pteroylmonoglutamic acid or folic acid; 5-formyltetrahydrofolic acid, 5-$HCOH_4$-PteGlu; 5-CH_3H_4-PteGlu, 5-methyltetrahydrofolic acid; H_4PteGlu, tetrahydrofolic acid; 10-HCO-folic acid, 10-formyl-folic acid.

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in the presence of substituents, and number of glutamyl residues attached to the pteroyl group. Naturally-occurring folates in reduced form are heat-labile and readily destroyed by oxidation. Heating in the presence of certain antioxidants, e.g. ascorbic acid, prevents the oxidation of folate. The most important dietary sources are fortified foods, liver and green vegetables. Up to approximately 80% of dietary folate exists in polyglutamated form (Herbert, 1990) and must be cleaved to the monoglutamate form prior to absorption and analysis.

The microbiological assay using *Lactobacillus rhamnosus* (formerly *Lactobacillus casei*) as the test organism (Keagy, 1985) is the most widely used method. This assay is considered both time consuming and demanding in execution despite development of semi-automated procedures (Horne & Patterson, 1988; Newman & Tsai, 1986). In addition, it only provides a total folate value of a food. The provision of analytical techniques to quantify the amount of folates in the diet has assumed increased importance for the reasons above. However, as a number of different folates occur naturally in foods, and PGA itself is now added to some foods, the amounts cannot necessarily be added together directly since they may be differently absorbed and metabolised. Recent studies have demonstrated that the absorption of folic acid in fortified foods is relatively high but the absorption of naturally-occurring folates may be much less (Cuskelly, McNulty, & Scott, 1996). Thus, there is a need to quantify each of the main folate forms separately.

In 1989, a project funded by the European Commission's Community Bureau of Reference (BCR) (in 1993 re-named as the Standard, Measurement and Testing programme) was initiated. The main objective was to improve the reliability and accuracy of methods for the determination of vitamins in food. The project included research into methodology (extraction and clean-up, end-method of determination, calibration), reducing the between-laboratory variation, and the preparation of suitably homogeneous food reference materials.

From the results of the first BCR intercomparison study which analysed folates in Brussels sprouts using microbiological methods, HPLC and radioassays (Finglas, Faure, & Southgate, 1993), it was concluded that additional work with the use of HPLC was required before these methods could be used routinely as an alternative to the microbiological assay. Five intercomparison studies for folates were completed for all folate methods over a seven year period (1990–1997) in which 21 participants from 13 countries took part. Five candidate reference materials (lyophilised Brussels sprouts powder, CRM 431; wholemeal flour, CRM 121; milk powder, CRM 421; lyophilised mixed vegetables, CRM 485, and lyophilised pig's liver, CRM 487) were studied. In addition, a yeast powder containing high concentrations of folates in the polyglutamate form was studied especially for the deconjugation step. One of the main objectives was to optimise the deconjugation step, especially the choice of γ-glutamyl hydrolase sources (human plasma, chicken pancreas, and hog kidney), pH, and length of incubation time used, sample clean-up prior to HPLC analysis, peak identification and calibration. The evaluation of current in-house procedures for food folate analysis using HPLC, with recommendations for the harmonization of procedures are presented in this paper.

2. Materials and methods

2.1. Participating laboratories

Nine laboratories, experienced in the use of HPLC procedures for the determination of folates in food, from six countries took part in the five studies (1990–1997). Not all laboratories took part in each study. It was agreed that each individual participant would meet the requirements of achieving the highest level of accuracy, precision and control of calibration during the work. Results were discussed at periodic evaluation meetings which nearly all participating laboratories attended.

2.2. Samples

2.2.1. Candidate reference materials

Lyophilised Brussels sprouts (CRM 431), was prepared by the lyophilisation of commercial, frozen Brussels sprouts, milled (<1 mm^2 particle size) and packaged into food-grade, heat-sealed laminate sachets under vacuum. The material had a residual moisture content of about 6%. Pig's liver (CRM 487) was a milled (<1 mm^2 particle size), lyophilised powder which had been prepared from fresh pig's liver, and filled into bottles under nitrogen, and sealed. It had a residual moisture of about 3.5%. Milk powder (CRM 421) was a spray-dried and vitamin enriched powder produced from cow's milk, and was packaged into sachets under nitrogen as above. The material had a residual moisture of about 3%. Wholemeal flour (CRM 121) was a commercially obtained wheat flour from a flour mill, packaged into sachets under nitrogen as above, and sealed. The material had a residual moisture of about 13%. Mixed vegetables (CRM 485) was a lyophilised mixture of sweet corn, carrot and tinned tomatoes (10:1:1, by weight). The vegetables were blended and the puree was lyophilised, milled (<1.5 mm^2 particle size) and packaged under nitrogen in sachets as above. All CRMs were stored at -30°C.

All reference materials were distributed to each participant by the same laboratory. Prior to distribution, both homogeneity, and short- and long-term stability,

for folate were assessed by microbiological assay, and found to be both homogeneous and stable (Finglas, Scott, Witthöft, van den Berg, & de Froidmont-Gö, in press).

A commercial milk powder sample was used for the second intercomparison study only.

To test the efficiency of the various batches of deconjugase enzymes, the total folate content of each batch of Bacto yeast powder (Difco Laboratories Ltd, Detroit, Michigan, USA) was determined by microbiological assay (Southon et al., 1994).

2.3. Enzymes (Table 1)

2.3.1. Pancreatic enzyme

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 enzyme solution was prepared as per a previous publication (Finglas et al., 1993). For the fifth study, participants included an α-amylase (e.g. Sigma) treatment for CRM 121 (wholemeal flour) prior to deconjugation.

2.3.2. γ-Glutamyl hydrolase enzymes (EC 3.4.22.12)

The hog kidney enzyme (HK) was prepared from fresh pig's kidneys as previously described (Gregory, Sartain, & Day, 1984). The activity of each batch of HK preparations was tested by determining total folate in the yeast extract above using a microbiological assay based on microtitration plates (Phillips & Wright, 1982; Southon et al., 1994). Lyophilised human plasma (HP; 5 ml) was obtained from Sigma (Poole, UK). Chicken pancreas enzyme (CP) was purchased from Difco (Detroit, MI) and prepared for use by Laboratory 7. Both HP and CP were prepared for use as previously described (Finglas et al., 1993).

2.4. Calibrants (Table 1)

Pteroylmonoglutamic acid (folic acid; PGA) was purchased from Sigma (Poole, UK) and (6R,S)-5-methytetrahydrofolic acid (5-CH_3H_4PteGlu; calcium salt) was obtained from Dr. B. Schircks (Jona, Switzerland). Sufficient quantities of these compounds were circulated to each participant for calibration purposes. PGA was prepared as previously described (Finglas et al., 1993). The actual concentration of the circulated 5-CH_3H_4PteGlu and the other in-house folate compounds (tetrahydrofolic acid, H_4PteGlu; 5-formyltetrahydrofolic acid, 5-$HCOH_4$-PteGlu; 10-formylfolic acid, 10-HCO-folic acid) were verified using the spectroscopic method as described in detail previously (van den Berg, Finglas, & Bates, 1994). The various molar extinction coefficients are given in Table 2, and are taken from Blakley (1969).

2.5. Design of studies

For each study, a protocol, including instructions for deconjugation using HK, HP and CP enzymes and preparation of calibration solutions, reporting sheets, and method questionnaires, samples and calibrants were sent out to each participant by courier. The aim of the first two studies was to establish the state-of-the-art and identify any specific problem areas for further work. The aim of the fifth (certification) study was to assign values for 5-CH_3H_4-PteGlu by HPLC, and total folate by microbiological assay, in the four candidate reference materials.

2.5.1. First intercomparison study

The participants were asked to follow the suggested extraction and deconjugation procedures as much as possible and determine the individual folate content of

Table 1
An overview of samples, deconjugase enzymes and calibrants used for each intercomparison study

Study no.	Samples	Deconjugase enzymes	Calibrants
I	Brussels sprouts powder (CRM 431)	HP, CP	None
II	Milk powder[a]	HP, CP + HK (in-house)	None
	Wholemeal flour (CRM 121)		
	Yeast powder		
III	Milk powder (CRM 421)	HK, HP + HK (in-house)	5-CH_3H_4-PteGlu
	Pig's liver (CRM 487)		PGA
	Wholemeal flour (CRM 121)		
IV	Milk powder (CRM 421)	HK + HP (in-house)	5-CH_3H_4-PteGlu
	Pig's liver (CRM 487)		
	Wholemeal flour (CRM 121)		
	Mixed vegetables (CRM 485)		
V	Milk powder (CRM 421)	HK + HK & HP (in-house)	5-CH_3H_4-PteGlu
	Pig's liver (CRM 487)		
	Wholemeal flour (CRM 121)		
	Mixed vegetables (CRM 485)		

[a] Milk sample used in study II was different from the vitamin enriched milk powder (CRM 421) used for studies III–V.

Table 2
Spectral data for folates on maximum absorbances and the molar extinction

Compound	Max λ (nm)	$E\%_{1cm}$ values (OD units μmol l^{-1} cm^{-1})
5-CH_3H_4PteGlu	290	31.7
5-$HCOH_4$PteGlu	285	37.2
10-HCO-folic acid	297	29.1
PGA	287	27.6
H_4PteGlu	297	29.1

Values taken from Blakley (1969).

Brussels sprouts using their normal in-house method. Both HP and CP enzymes together with pancrex capsules were circulated.

2.5.2. Second intercomparison study

Three materials [a milk powder sample (3×50 g), wholemeal wheat flour (CRM121; 3×50 g), and yeast powder (4×20 g)], HP and CP enzyme preparations, were all supplied. The use of any in-house deconjugase was optional. The choice of the HPLC method was left to the individual participants (see Table 3). A calibration graph and a labelled, representative chromatogram of both standards and samples (before and after deconjugation with various incubation times), together with appropriate evidence of peak identity, were also requested.

2.5.3. Third intercomparison study

Lyophilised HK and HP were supplied for deconjugation; results obtained using any other in-house deconjugase enzyme, which were found to be acceptable, could be reported. Enzyme blanks were also to be reported. In order to check in-house calibrations, a 5-CH_3H_4PteGlu standard was supplied with instructions for preparation of stock calibration solutions (see calibrants above). Two candidate reference materials (CRM 421, a vitamin enriched milk powder; CRM 487, a lyophilised pig's liver) were introduced for the first time.

2.5.4. Fourth intercomparison study

For this study, mixed vegetables powder (CRM 485; 3×25 g) was included for the first time. For deconjugation, HK was only supplied. Any in-house deconjugase (including HP) could also be used in addition to the circulated enzyme. The concentration of the circulated 5-CH_3H_4-PteGlu was to be determined as before and recovery data for 5-CH_3H_4PteGlu was to be reported before and after deconjugation. A recommended minimum sample size of 2–5 g was given. It was stressed that forms other than 5-CH_3H_4PteGlu may also be reported providing that participants were satisfied with method validation.

2.5.5. Fifth intercomparison (or certification study) study

Emphasis was placed on the determination of the 5-CH_3H_4PteGlu concentration before and after deconjugation using circulated HK. Each participant was selected on the basis of their performance in previous studies and was required to provide results from at least five replicate analyses on two separate sachets of each of four candidate reference materials, performed on different days. The use of ascorbic acid instead of sodium ascorbate during extraction was included and a target recovery of at least 75% for 5-CH_3H_4-PteGlu added prior to extraction and deconjugation was set. Other folate forms could also be reported if participants were satisfied with their methodology, especially the validation, e.g. recovery data, peak identification (spiking, spectral scanning), peak resolution, peak purity, and calibration. However, if time was limiting, participants were asked to determine 5-CH_3H_4-PteGlu only.

2.6. Sample extraction and deconjugation procedures

Participants were requested to use their own in-house extraction procedures during each of the studies (Table 3). The deconjugation system for circulated HK consisted of sample extract (1 ml), reconstituted HK preparation (0.5 ml), and extraction buffer (pH 4.5; 3.5 ml). The buffer consisted of 0.1 M sodium phosphate and 1% ascorbic acid. The mixture was incubated at 37°C for 3 h followed by heating at 100°C for 5 min in order to inactivate the enzymes. The whole digest was cooled, transferred to a volumetric flask, and made up to 10 ml volume with buffer. The sample extracts were centrifuged (1000×***g***) for 10 min and the supernatants were removed and stored at −18°C in a plastic tube prior to analysis. An enzyme blank solution was also included. The procedures for deconjugation using HP and CP were the same as previously described (Finglas et al., 1993).

2.7. Methods used

A summary of the HPLC methods used are given in Table 3. The various in-house microbiological methods used to obtain the certified values for total folate in each of the CRMs (Table 9) are reported elsewhere (Finglas et al., in press).

3. Results and discussion

3.1. Intercomparison studies I and II

The results of the two participants who completed the first study are given in Fig. 1. Laboratory 1 was able to report H_4PteGlu, 5-CH_3H_4PteGlu, and

Table 3
Details of the HPLC procedures used for the intercomparison studies

	Lab 1	Lab 2	Lab 3	Lab 3a	Lab 4	Lab 5	Lab 6	Lab 7	Lab 8	Lab. 9
Sample size (g)	2	0.5	2–10	1–8.5	1.5	1.5–2.5	1–5	2	2.5–5	2
Extraction	0.05 M AB + 1% Na asc (pH 4.9); 5 min/100°C; centrifuged; HK, 2 h/37°C;	0.1 M AB + 1% AA (pH 4.5); 15 min/100°C, nitrogen flush; HK/HP pH 4.5, 3 h, 37°C, nitrogen flush; 5 min/100°C	0.1 M PB + 0.4% AA (pH 7.4); 10 min/100°C, nitrogen flush; HK/HP, pH 4.5, 3 h, 37°C; 5 min/100°C	0.1 M PB + 1% AA (pH 6); 10 min/100°C, nitrogen flush; HK/HP, pH 4.5, 3 h, 37°C; 5 min/100°C	0.1 M AB + 1%AA (pH4.5), 15 min/100°C, nitrogen flush; 5min/100°C	0.075 M PB + 1% AA & MCE (pH 6), 1 min microwave + 10 min/100°C, nitrogen flush, HK/HP, pH 4.5, 3 h/37°C, 5min/100°C	0.1M PB + 2% AA (pH 7), centrifuge, HK/HP, 3 h/37°C	0.1M PB + 0.1% AA (pH 6), 5 min/121°C, centrifuge, HK, pH 4.5, 3 h/37°C, 5min/100°C, centrifuge	0.1 M PB + 0.1% AA (pH 6), HK (pH 4.5), 3 h/37°C, 5 min/100°C; centrifuge	0.1 M PB + (0.1% AA, pH 6), 5 min/121°C, centrifuge, HK (pH 4.5), 3 h/37°C, 5 min/100°C, centrifuge
Clean-up	SAX (DEAE-Sephadex A25)	None	Filter (0.45μm)	SAX (Merck)	SAX (Bond Elut)	SAX (Baker)	None	SAX (Bond Elute)	SAX (Baker)	SAX
HPLC Column	μ-Bondapak C18 (150×4 mm) or μ-Bondapak phenyl 10 μm (300×4 mm) (both Waters)	Spherisorb 5 μm (250×5 mm) (Phase Separation)	ODS Hypersil 5μm (250× 4 mm) (Gynkotek)	Merck RP-18 5μm (125× 4 mm)	Merck RP18 (250×4 mm)	Spherisorb 5 μm (250×4.6 mm) or Hypersil ODS 3 μm (150×4.6 mm) (Shandon)	Nova-Pak phenyl (150×3.9 mm)	PLRPS (250×4.6 mm)	Vydac 201 TP 54, RP18 (250×4.6 mm)	Waters Nova-Pak C18 (100×8 mm)
Mobile phase	0.033 M KH_2PO_4: CH_3CN (90:10), pH 2.3; 1 ml min^{-1}	H_2O:CH_3CN (90:10), pH 2.3, 1 ml min^{-1}	Water:MeOH (70:30) + 5 mM TBAP, pH 7.4, 1.5 ml min^{-1}	0.1 M H_3PO_4 (pH 5.1): CH_3CN:MeOH (86.5:11:2.5) + 5 mM TBAP, 1 ml min^{-1}	ACAC (6%): CH_3CN (92:8), 0.4 ml min^{-1}	PB (30 mM, pH 2.2): CH_3CN (gradient 9–24% CH_3CN in 9 min, 30°C	0.05M KH_2PO_4: MeOH (85:15) + EDTA, pH 3.5	0.03 M PB (pH 2):CH_3CN, gradient, 1 ml min^{-1}	0.033 M PB (pH 2.2): CH_3CN, gradient, 0.8 ml min^{-1}	0.033 M PB (pH 2.3): CH_3CN (93:7), 1 ml min^{-1}
Detection	F at 292/356 nm	F at 296/365 nm	uv at 280 nm	F at 280/365 nm	F at 310/352 nm	F at 290/356 nm and 360/460 nm (10-$HCOH_4$-PteGlu only); uv at 290 nm (5-$HCOH_4$-PteGlu and PGA)	F at 295/365 (H_4PteGlu and 5-CH_3H_4-PteGlu) & 295/372 nm (5-$HCOH_4$-PteGlu)	F at 300/360 nm	F at 280/359 nm (5-CH_3H_4Pte-Glu and H_4PteGlu)	F at 295/356 nm
Peak identification	RT standards	RT standards	RT standards	RT standards + spiking	RT standards + spiking	RT standards, spiking, dual detection	RT standards, spiking	RT standards, spiking	RT standards, spiking	None
Reference	Gregory et al. (1984)	Farrar et al. (1992)	Schulz, Weidemann, and Bitsch (1985)	Witthöft and Bitsch (1993)	Wigertz and Jagerstad (1995)	Vahteristo et al. (1996a)	Lucock et al. (1995)	(unpublished)	Gregory et al. (1984)	Gregory et al. (1984)

AB, acetate buffer; AA, ascorbic acid; F, fluorescence detection; PB, phosphate buffer; SAX, strong anion exchange column; SPE, solid phase extraction; TBAP, tetrabutyammonium phosphate ion-pair; uv, ultra-violet detection.

5-$HCOH_4$-PteGlu, whereas laboratory 2 initially only found 5-CH_3H_4PteGlu. Low levels of H_4PteGlu (15–20% of 5-CH_3H_4PteGlu) were identified later by laboratory 2. This laboratory seemed to report considerably more 5-CH_3H_4-PteGlu compared to the other laboratory. Both laboratories used reverse phase systems with fluorometric detection but laboratory 2 omitted the clean-up stage. Chromatograms from laboratory 1 contained fewer non-folate peaks compared to laboratory 2. Laboratory 3a used a reverse phase system with an ion-pair reagent and UV detection at 280 nm and no sample clean-up. They were not able to report any results due to poor baseline resolution of folate peaks with this system (Finglas et al., 1993). All three deconjugase enzymes (HP, CP and in-house HK) gave similar folate concentrations in Brussels sprouts samples.

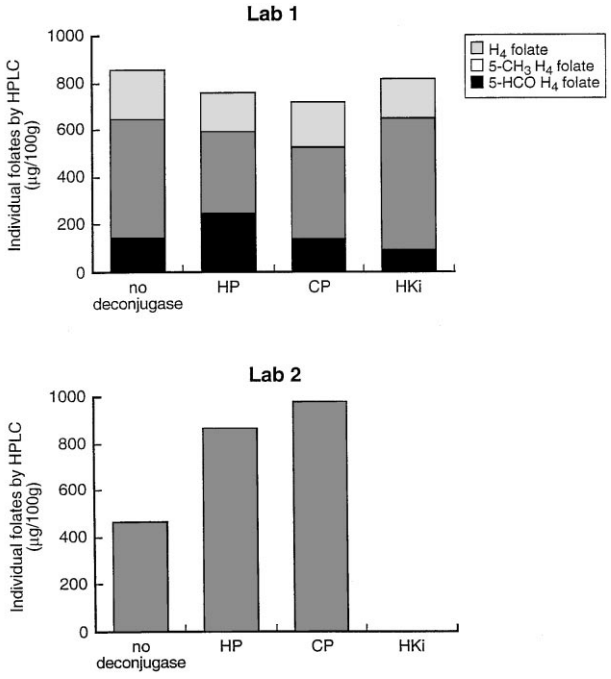

Fig. 1. Individual folates (µg/100 g) in lyophilised Brussels sprouts powder (CRM 431) using HPLC reported by laboratories 1 and 2 (1st study). Results are with no deconjugation, and following deconjugation using circulated human plasma (HP) and chicken pancreas, and in-house hog kidney (HKi) enzymes. See Table 3 for full details of procedures.

The results for the two participants who completed the second study with milk powder, wholemeal flour, and yeast are summarised in Fig. 2. Laboratory 3a took part again using an improved reverse phase system with UV detection which gave baseline separation of H_4PteGlu, 5-$HCOH_4$-PteGlu and 5-CH_3H_4-PteGlu. Laboratory 4 took part for the first time. The folate patterns differed greatly between the two participants for each of the three samples. Laboratory 4 consistently reported 5-CH_3H_4-PteGlu but this form was not found by laboratory 3a who mainly found H_4PteGlu. In view of the low concentrations of these reduced folates and the weak UV-absorbing nature of these compounds, it was concluded that fluorometric detection was necessary for quantitation.

The main conclusions from these two initial studies was that (1) sample clean-up was required prior to HPLC analysis with fluorometric detection, (2) UV detection was not sufficiently sensitive to determine natural folate concentrations in these samples, (3)

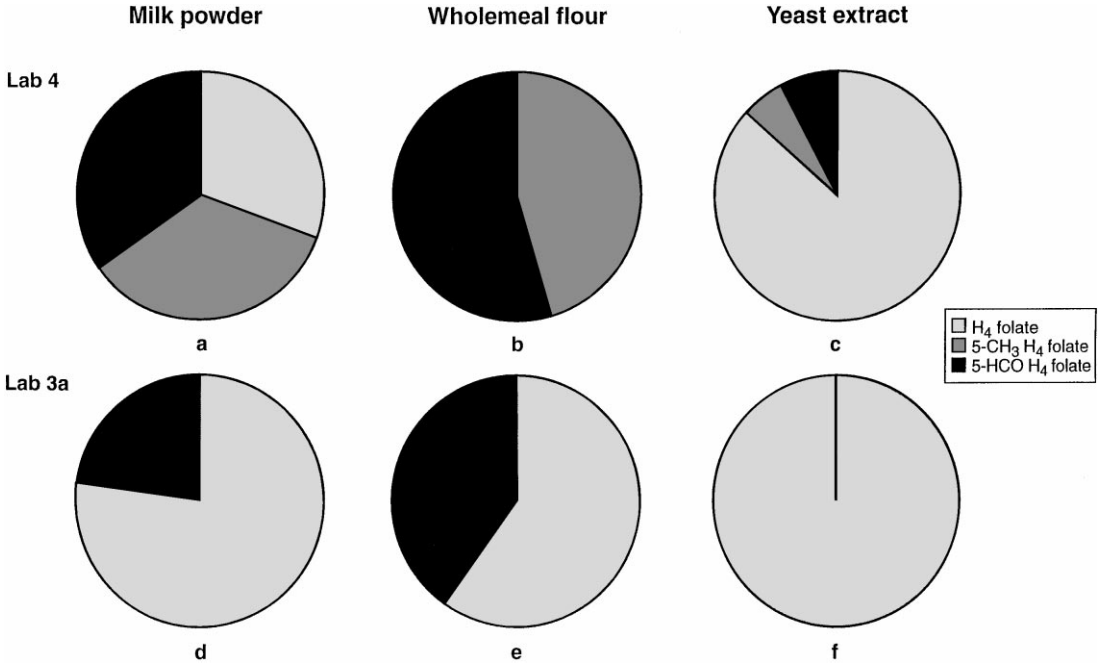

Fig. 2. Average distribution of folates (%) reported by laboratories 3a and 4 in milk powder (a and d), wholemeal flour (CRM 121; b and e), and yeast powder extract (c and f) following deconjugation using HP (2nd study). See Table 3 for full details of procedures.

further work on peak identification and calibration procedures were required, and (4) additional laboratories needed to be recruited.

3.2. Intercomparison studies III–V

The number of laboratories who took part in these studies rose from three in study III to six in study V. All laboratories now used reverse phase systems with fluorimetric detection (Laboratory 6 reported the sue of electrochemical detection as well) and some form of sample clean-up prior to chromatographic separation (Table 3). These procedures are largely based on a previously published HPLC method for the determination of folates in foods and biological samples (Gregory et al., 1984).

Greater attention was given to the preparation of stock calibration solutions and concentrations were verified using molar extinction coefficients (Table 2). The type, source and purity of the various folate calibrants used are given in Table 4. The purity of PGA was consistently found to be close to 100% but typical purity values for 5-CH_3H_4-PteGlu (from Schircks) were around 80%. All folate values were corrected for purity of stock 5-CH_3H_4-PteGlu standard for each of these studies. The purities of H_4PteGlu and 5-$HCOH_4$-PteGlu varied between 70 and 90% depending on the source used.

3.2.1. Between-laboratory agreement for the determination of individual forms

The individual folate results using the common HK deconjugase enzyme for the four candidate reference materials are given in Table 5 for each of the studies. The most consistently reported values were for 5-CH_3H_4-PteGlu, and to a lesser extent, H_4PteGlu. Participants reported most difficulty with the extraction and clean-up of the wholemeal flour samples. These were the lowest in folate concentration and contained some interferences in the chromatographic separation. All participants in the fifth study were asked to include an α-amylase treatment prior to deconjugation for the wholemeal flour sample in order to remove starch and

Table 4
Type, source and purity of various folate calibrants used in studies III–V

Compound	Type	Source	Purity (%) as measured using molar extinction coefficient (no. labs)
Study III:			
PGA	Free acid	Merck/Sigma	>99 (2)
5-CH_3H_4-PteGlu	CA salt	Schircks	82 (3)
H_4PteGlu	Free acid[a]	Sigma	78 (2)
H_4PteGlu	Free acid[a]	Fluka	93 (1)
5-$HCOH_4$-PteGlu	CA salt	Sigma	69 (2)
5-$HCOH_4$-PteGlu	CA salt	Fluka	79 (1)
Study IV:			
5-CH_3H_4-PteGlu	CA salt	Schircks	78 (7)
5-CH_3H_4-PteGlu	CA salt	Schircks	81 (5)

[a] Contains 0.1% mercaptoethanol.

Table 5
Individual folate results (μg/100 g dry matter) for four candidate reference materials obtained by HPLC following hog kidney deconjugation using common enzyme preparation in the 3rd–5th intercomparison studies (results are means with ranges in parentheses)

Study no.	Sample	*n*	5-CH_3H_4-PteGlu	*n*	H_4PteGlu	*n*	5-$HCOH_4$-PteGlu	*n*	10-$HCOH_4$-folic acid	*n*	PGA
III	Milk powder	3	36 (29-49)	3	23 (6-55)	1	70	–	–	1	68
IV	Milk powder	4	17 (10-31)	2	7 (3-11)	–	–	–	–	1	71
V	Milk powder	6	25 (17-32)	–	–	–	–	–	–	–	–
III	Pig's liver	3	203 (64-284)	3	328 (95-595)	2	400 (156-643)	1	36	–	–
IV	Pig's liver	4	309 (27-542)	3	467 (334-484)	1	146	1	37	1	139
V	Pig's liver	5	215 (186-353)	–	–	–	–	–	–	–	–
III	Wholemeal flour	2	19 (4-33)	2	5 (3-7)	–	–	–	–	–	–
IV	Wholemeal flour	3	16 (7-26)	2	10 (7-13)	1	427	1	6	–	–
V	Wholemeal flour	5	7 (2-19)	–	–	–	–	–	–	–	–
IV	Mixed vegetables	4	294 (156-557)	1	5	–	–	–	–	–	–
V	Mixed vegetables	5	202 (167-229)	–	–	–	–	–	–	–	–

n = Number of results.
"–" = Not reported.

aid complete extraction of folate. Several workers have suggested α-amylase and protease enzymes, in addition to the deconjugase enzymes, for extraction of folates from some foods, mainly cereals and cereal products (Desouza & Eitenmiller, 1990; Pfeiffer, Rogers, & Gregory, 1997). The latter workers have recently shown that tri-enzyme treatment gave between 4 and 33% increase in measurable folate in white bread, spaghetti and rice.

Laboratory 5 reported values for PGA in milk powder using fluorescence and subsequent uv detection at 290 nm. This folate form was used for enrichment purposes in this material. The sum of the individual folates in milk powder and pig's liver reported by laboratory 5 for the third study agreed very favourably with total folate values obtained by microbiological assay (Fig. 3). This demonstrated that, for some foods it is possible to use HPLC procedures to provide total folate values, although it is advisable to provide microbiological data as well as part of the validation of peak identification and quantification in the HPLC procedure used.

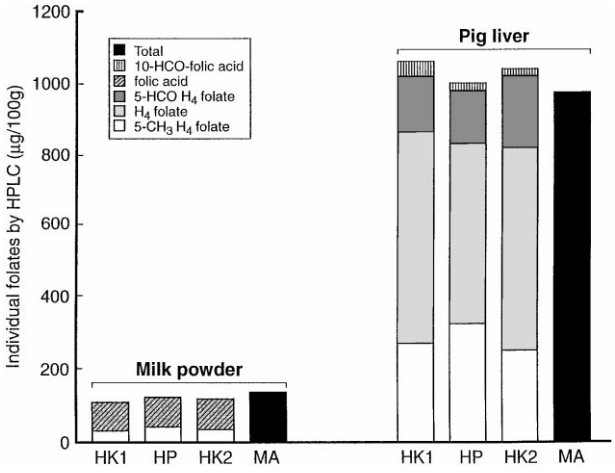

Fig. 3. Individual folate vitamers in milk powder (CRM 421) and pig's liver (CRM 487) reported by laboratory 5 in 3rd study following three deconjugase enzymes, and total folates determined by microbiological assay (MA). HK1, circulated hog kidney; HK2, in-house hog kidney; HP, circulated human plasma. See Table 3 for full details of procedures. [Adapted from Vahteristo et al., *Food Chemistry, 57*(1), 109–111, 1996b].

A comparison of the between- and within-laboratory agreement for the determination of 5-CH_3H_4-PteGlu by HPLC and total folate by MA in the four candidate reference materials obtained in the 5th (certification) study is given in Table 6. The HPLC variability compares very favourably to the MA and can now be used with some confidence for 5-CH_3H_4-PteGlu analysis in food. Further work may be necessary for the other folates. Although, good agreement between the sum of the individual folate forms by HPLC and total folate by MA can be found for some foods (see Fig. 3 above), the latter should still be regarded as the most appropriate value for inclusion into food composition tables. Values for individual folates by HPLC (mainly 5-CH_3H_4-PteGlu) can also be presented for selected foods because these data are useful, for instance, for bioavailability studies and effects of processing on retention of different forms.

The between-laboratory agreement found in the fifth certification study (3–34% for HPLC & 14–23% for MA) is somewhat less than two previous reported studies for the determination of folate in serum or whole blood. The latter reported between-laboratory variations of 18–41% and 25–74%, respectively, using a combination of radioassays and MAs (van den Berg et al., 1994), and 28 and 36% using radioassays, MA and HPLC procedures (Gunter et al., 1996). The analysis of folates in blood should be easier than food because 5-CH_3H_4-PteGlu (monoglutamate and polyglutamate forms) is the predominant form present. However, despite this there appears to be greater inter-method variation for the determination of folates in blood. The higher between-laboratory variation for the determination of 5-CH_3H_4-PteGlu in pig's liver (CRM 487) may be due to varrying degrees of complete deconjugation. One laboratory reported that a longer incubation time (4–5 h) was needed for complete deconjugation instead of the 3 h suggested.

3.2.2. *Effect of different deconjugase enzymes*

Four deconjugase enzymes were used during the course of this work: a common HK enzyme prepared from fresh pig's kidneys using a previously described procedure (Gregory et al., 1984), various in-house HK enzymes, various CP preparations (for MA only), and

Table 6
Summary of the within-laboratory (%CV_W) and between-laboratory (%CV_B) variation for 5-CH_3H_4-PteGlu by HPLC and total folate by microbiological assay (MA) in four candidate reference materials from the certification (5th) study

Reference material	CRM no.	HP LC		Microbiological assay (MA)	
		CV_W (%)	CV_B (%)	CV_W (%)	CV_B (%)
Wholemeal flour	121	–	–	9.2	22.3
Milk powder	421	4.7	2.7	6.7	16.9
Mixed vegetables	485	4.9	7.6	4.9	13.8
Pig's liver	487	7.1	33.9	14.8	17.0
		Mean: 5.6	Mean: 14.7	Mean: 8.9	Mean: 17.5

Table 7
Concentrations of 5-CH_3H_4-PteGlu) (μg/100 g dry matter) in four candidate reference materials using different deconjugase enzymes. Results are means with ranges in parentheses

Study no.	Sample	Deconjugase enzyme	*n*	5-CH_3H_4-PteGlu
III	Milk powder	HK (common)	3	36 (29–49)
		HK (in-house)	1	30
		HP	2	29 (21–37)
IV	Milk powder	HK (common)	4	17 (10–31)
		HK (in-house)	2	29 (26–31)
		HP	3	25 (17–31)
III	Pig's liver	HK (common)	3	203 (64–284)
		HK (in-house)	2	218 (165–270)
		HP	2	221 (105–336)
IV	Pig's liver	HK (common)	4	309 (57–542)
		HK (in-house)	2	344 (286–401)
		HP	3	304 (140–271)
III	Wholemeal flour	HK (common)	2	19 (4–33)
		HK (in-house)	2	21 (4–35)
		HP	2	20 (7–33)
IV	Wholemeal flour	HK (common)	3	16 (7–26)
		HK (in-house)	2	14 (4–23)
		HP	3	15 (3–27)
IV	Mixed vegetables	HK (common)	4	294 (156–557)
		HK (in-house)	2	182 (168–196)
		HP	3	173 (131–196)
V	Mixed vegetables	HK (common)	5	202 (167–229)

n, Number of results; HK, hog kidney deconjugase enzyme and HP, human plasma deconjugase enzyme.

commercially available lyophilised human plasma (HP). The activity of each batch of enzyme was checked by measuring total folate in a commercial yeast preparation and found to vary no more than 5–10% between batches. There was little endogeneous folate present in this preparation and the enzyme activity was stable for at least 6 months at −18°C. Both these enzyme types (HK and HP) give essentially monoglutamate folate end-products which are necessary for HPLC separation and quantification (Finglas et al., 1993).

The effect of the three different deconjugase enzymes on folate concentrations in the four candidate reference materials are given in Table 7. In general all enzymes gave similar folate values with the possible exception of in-house HK and HP in mixed vegetables (ca. 39% lower) vs common HK, and common HK in milk powder (mean value ca. 59% lower) versus other two enzymes. There is no single source of deconjugase enzyme which is commercailly available so that it is important for laboratories to obtain the same folate values using in-house enzymes as well as a common circulated enzyme preparation. In addition to these enzymes, chicken pancreas can also be used for MA work but not for HPLC (Finglas et al., 1993).

3.2.3. Recovery of 5-CH_3H_4-PteGlu

After the third study, it was agreed the recovery of 5-CH_3H_4-PteGlu added prior to sample extraction and clean-up should be measured and optimised as fully as possible. Although the recovery of added vitamin does not provide any information regarding losses or interconversions of endogenous folates during extraction and deconjugation, it is a useful part of the overall validation procedure.

Table 8
Recovery data for 5-CH_3H_4-PteGlu for IV and V studies. Results are means with ranges in parentheses

Study no.	Sample	*n*	Recovery (%)
IV	Milk powder	3	78 (55–91)
V	Milk powder	6	84 (59–119)
IV	Pig's liver	4	86 (73–105)
V	Pig's liver	5	93 (70–111)
IV	Wholemeal flour	3	64 (52–83)
V	Wholemeal flour	6	95 (69–127)
IV	Mixed vegetables	3	72 (55–83)
V	Mixed vegetables	5	93 (70–140)
			Mean: 75 (4th)
			Mean: 91 (5th)

The recovery data for the fourth and fifth studies are given in Table 8. There was a general improvement in recovery of 5-CH_3H_4-PteGlu from the fourth study to the certification study of about 16% with the largest increases being found for the wholemeal flour and mixed vegetable samples. The latter were only introduced for the fourth study onwards. The introduction of the α-amylase treatment in addition to the deconjugase may have contributed to the much improved recovery found for this CRM in the certification study. A target recovery for 5-CH_3H_4-PteGlu of 75% was set for the certification study for laboratories using HPLC procedures and all laboratories were able to achieve this.

3.2.4. Proposed certified, indicative and information values for total folate and 5-CH_3H_4-PteGlu in four candidate reference materials

The aim of the fifth (certification) study was to assign certified values for total folate in four candidate reference materials (wholemeal flour, CRM 121; milk powder, CRM 421; lyophilised mixed vegetables, CRM 485 and lyophilised pig's liver, CRM 487. These CRMs will be used primarily for the quality control of analytical measurements for nutritional labelling, and the development and validation of alternative procedures. Both the stability (short- and long-term) and homogeneity of folate in these materials have been fully evaluated and found to be satisfactory. The results are presented elsewhere (Finglas et al., in press). The participants that took part in the certification study were chosen on the basis of their performance in previous studies.

The proposed certified values for total folate (by MA), and indicative and information values for 5-CH_3H_4-PteGlu (by HPLC) in the four candidate

Table 9
Proposed indicative and information values for 5-CH_3H_4-PteGlu, and certified values for total folate by microbiological assay, in four candidate reference materials. Values are expressed as mg kg^{-1} dry matter with uncertainties of measurement, and number of accepted results, in parentheses

Reference material	CRM no.	Certified values for total folate[a]	Indicative values for 5-CH_3H_4-PteGlu[a]	Information values for 5-CH_3H_4-PteGlu[b]
Wholemeal flour	121	0.50 (0.07; 15)	–	0.04 (0.03–0.08; 2)
Milk powder	421	1.42 (0.14; 15)	0.25 (0.02; 4)	–
Mixed vegetables	485	3.15 (0.28; 12)	2.14 (0.42; 3)	–
Pig's liver	487	13.3 (1.3; 17)	–	2.6 (1.9–3.8; 3)

[a] Taken as the average of the data set averages with the uncertainty (half-width of the 95% confidence interval of the data set averages).
[b] Taken as the average of the data set averages with the range, and number of accepted results, in parentheses.

reference materials are given in Table 9. The data sets were evaluated statistically using a number of tests in order to check (1) if the population of results accepted had a normal distribution before using the 95% confidence interval of the mean of means, and (2) to assess if the data from all methods and laboratories can be considered as one homogeneous set and pooled. A full description of the statistical results are presented elsewhere (Finglas et al., in press).

There was considerably more accepted data sets for total folate by MA compared to 5-CH_3H_4-PteGlu by HPLC. It was agreed that the level of agreement for total folate was sufficient to permit draft certified values be proposed for each of the CRMs. The level of the uncertainty was in each case calculated to be ca. 10% of the certified value. Draft indicative values only were proposed for 5-CH_3H_4-PteGlu in CRMs 421 and 485 due to the limited data available. It was felt that for CRMs 121 and 487, only information values (with ranges) could be proposed for the following reasons. For CRM 121, all laboratories reported problems with extraction and the presence of interferring peaks due to the low folate concentrations present. For CRM 487, there was some indication that deconjugation was not complete and a longer deconjugation incubation time was needed (ca. >4 h). It was felt that any additional folate data for CRM 121 was important with the mandatory fortification of certain cereal products in the USA being introduced from 1 January, 1998, which will require additional folate analyses by regulatory and other enforcement laboratories.

4. Conclusions

These results clearly illustrate the improvements in the determination of individual folates over a series of intercomparison studies. Through careful validation and detailed discussions held at evaluation meetings after each intercomparison study, possible biases and sources of systematic error have been identified and reduced. The use of sample clean-up is crucial in order to maintain high reliability and the use of spectrometrically calibrated standards is essential. Four candidate reference materials with certified values for total folate by MA and indicative/information values only for 5-CH_3H_4-PteGlu by HPLC have been proposed. The availability of these CRMs will greatly assist in the quality control of folate measurements for nutritional labelling, and validation of alternative techniques. Further methodology work is needed for HPLC analysis of folate forms other than 5-CH_3H_4-PteGlu especially stability during extraction and sample clean-up, and possible interconversions between forms during deconjugation.

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