The determination of folate in food using HPLC with selective affinity extraction

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The determination of folate in food using HPLC with selective affinity extraction

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Executive Summary

Folate is the generic name for pteroyl glutamate compounds that exhibit the biological activity of folic acid. Folic acid itself (pteroyl glutamate) is not found naturally in foods but is the main commercially-available synthetic form and is the USP standard. It is often used for fortification of foods and supplements. Naturally occurring folates are reduced and substituted pteroyl polyglutamates containing typically between 5 & 7 glutamate moieties.

A full analysis of the folate profile of foods requires identification and determination of the different folate forms present however, methods used for labelling purposes typically only measure the total folate content. The aim of this project was to develop and evaluate a procedure for the determination of both natural and added folates using HPLC.

Foods can contain a number of different folate forms although the most significant compounds are 5-methyl H_4folate, 5CHO-H_4folate, H_4folate and when added, folic acid itself. The HPLC conditions required to separate these compounds have been identified and suitable calibration ranges established for each compound of interest.

Food extracts must be purified and concentrated prior to HPLC and this was accomplished using a non-proprietary affinity medium containing folate binding protein. Folate extraction cartridges were prepared and were tested for their ability to retain the different folate forms. All of the target forms were retained by the cartridges as evidenced by the recovery of folates from standard solutions. Each cartridge had a nominal capacity for folate of ~ 8µg however; this was restricted to 1µg for routine use to ensure that the capacity was not exceeded.

A range of test foods were analysed for their folate content using a tri-enzyme extraction followed by sample clean-up using the affinity cartridges and determination by HPLC.
The folate forms present were identified and quantified and the sum of these was compared to the expected values for total folate. The results obtained agreed quite well with the expected values for total folate. Unfortunately, the affinity media proved to be unstable when used for sample extracts and results for subsequent assays where the cartridges were reused were low and variable.

The method can be used for the determination of natural and added forms of folate in foods when information about the types of folates present is required. The instability of the affinity cartridges and the time and cost of preparing these however, limits the usability of the procedure for routine use.

1. Background

1.1 Folates in food

Folate is the generic name for pteroyl glutamate compounds that exhibit the biological activity of folic acid. Folic acid itself (pteroyl glutamate) is not found naturally in foods but is the main, commercially-available synthetic form and is the USP standard. It is often used for fortification of foods and supplements.

Naturally occurring folates are reduced and substituted pteroyl polyglutamates containing typically between 5 & 7 glutamate moieties.

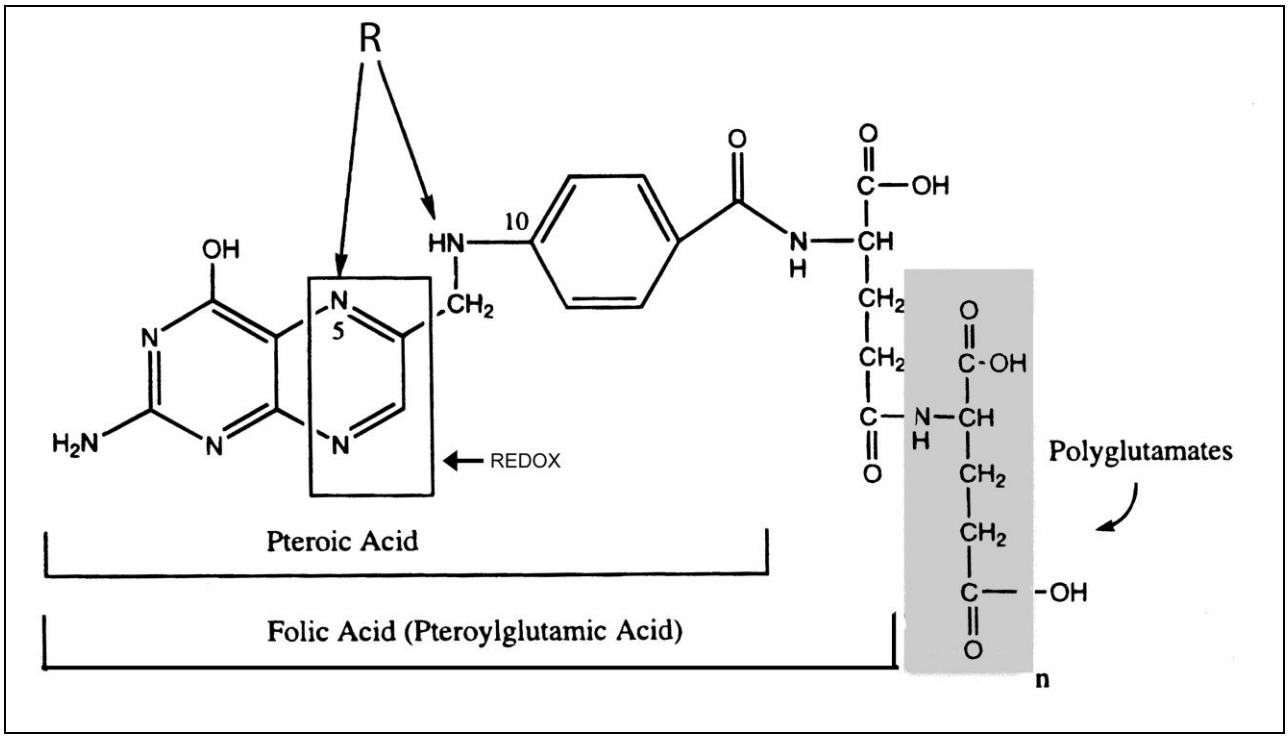

Figure 1: Structure of folates

The main circulatory form in humans is 5-methyl tetrahydrofolate (5MeH4 folate). This form is available commercially and is also used for fortification of foods. Other forms of folate are found in foods or as metabolites in biological fluids. These forms vary in the state of oxidation of the pteridine ring and in the substituents linked at the 5 & 10 positions. Their biological activity varies.

Examples are shown below:

Table 1: Forms of folate

Name	Abbreviation
5 methyl tetrahydrofolate	5MeH_4folate
5formyl tetrahydrofolate	5CHOH_4folate
10formyl tetrahydrofolate	10CHOH_4folate
5,6,7,8 tetrahydrofolate	H_4folate
7,8 dihydrofolate	H_2folate
5 methyl dihydrofolate	5MeH_2folate
5, 10 methenyl tetrahydrofolate	5, 10, CH^+=H_4folate
5, 10 methylene tetrahydrofolate	5,10-CH2H_4folate

A full analysis of folate profiles for clinical research purposes, may require identification of all of these forms, together with the number of glutamate molecules attached to the pteroyl structure and can be very complex however such analysis is not normally required for the estimation of total folate activity from food. The length of the polyglutamate chain primarily affects the rate and degree of absorption of folate in the gut and although important for bioavailability studies, it is not necessary to know this for estimation of activity in food. It is only necessary to ensure that the folates are in a form that can be determined by the analytical method used. The polyglutamate chain is usually cleaved enzymatically during extraction in order to simplify subsequent analysis.

1.2 Fortification:

Folate is regarded as a water soluble vitamin (sometimes referred to as Vitamin B_9). Deficiency of folate is prevalent in many areas of the world and causes anaemia and a range of other health problems. Deficiency can result from poor dietary intake, defective absorption, abnormal metabolism or clinical therapy. In women of child-bearing age, lack of folate can cause neural tube defects in the developing foetus and this is the primary reason for mandatory fortification programmes used in several countries. The UK is still considering whether such a programme should be mandated in the UK. Folic acid or 5-methyl H_4 folate is used for fortification. Voluntary fortification of food is also widely practiced, mainly using folic acid.

1.3 Analysis of folates

Analysis of folate is necessary to support food labelling requirements, nutritional claims, for nutritional studies, or for clinical/ medical studies. The latter generally requires more extensive

and specific analysis of folate compounds and is principally carried out in blood, sera and other body fluids.

For food labelling and food control purposes, it is normally sufficient to determine the total folate content of the food but this may require determination of individual forms depending on the method chosen. For fortified foods, it is usually only necessary to determine the amount of the added form of folate present since the amount of naturally present folates is not normally significant in such foods.

For unfortified foods, the total folate activity is determined or the different forms present are quantified individually and summed to calculate the total activity. This can be necessary for nutritional studies or where claims are made for unfortified products.

1.4 Analytical Methods

Total folate can be determined using microbiological assay, ligand binding assay or radioimmunoassay. The latter two techniques are most often used for clinical determination of folate but microbiological assay is still used for determination of folate in food. A number of modifications have been made to the microbiological assay to improve method reliability and to reduce variability. This procedure only provides information about the total folate content unless complex, differential assays are carried out.

Microbiological methods are still included in compendium methods such as those from the AOAC but increasingly, instrumental methods are used. Folates in food can be determined using HPLC with UV or fluorescence detection and LC-MS procedures are becoming available. Such procedures can distinguish between individual folate forms. Folate analysis is complicated by the number of different forms which must be analysed, the varying length of the glutamate side-chain and the generally poor stability of folates. These factors must be considered when evaluating the scope, specificity and performance of any analytical method.

A commercially available microbiological assay test kit (Vitafast®) is available for the determination of total folate. This kit removes the need to maintain microbiological organisms in the laboratory and simplifies the analytical procedure. It is however, still subject to the effects of chemical or bacteriological contamination and must be used with appropriate care. An immunoaffinity extraction cartridge is also available which can be used to selectively extract folic acid from foods for analysis by HPLC however; this does not recover other forms of folate. It should be noted that these commercial kits provide guidance on the extraction procedures

required but that they may not always be relevant for every sample type. Some additional within-laboratory validation is often required.

In order to determine the folates found naturally in foods or to correctly calculate the total folate content, determination of the separate forms is required. Any method for the determination of folates must consider the following points:

- Scope of the method
- Sample pre-treatment
- Sample extraction
- Analyte stability
- Removal of interfering components
- Method of detection

1.4.1 Scope of the method:

Analysts must decide whether they are required to measure total folate or individual folate forms. Much of the current data available in national food tables was obtained using microbiological assay. Where more specific procedures are used, the total folate can be calculated by summing individual forms however, there is often a difference between these figures. The reasons for such differences need to be considered. Where knowledge of the folate profile is required, selective, instrumental methods must be used.

1.4.2 Sample pre-treatment

Folates are labile and can degrade during storage, processing and analysis. Samples should be stored in their original state and for as short a time as possible. Generally samples may be frozen or freeze-dried if analysis cannot be carried out immediately, however it is possible for some losses of folate to occur. Checks should be carried out if significant losses are suspected. Once sample maceration is carried out, analysis should proceed as quickly as possible.

1.4.3 Sample Extraction:

The extraction procedure used is key to the reliable estimation of folate from foods.
Added folate can be determined after simple aqueous extraction, normally with heating. Some sample clean-up or concentration is often required. Antioxidants such as ascorbic acid should be used to protect the labile folates during extraction and subsequent analysis.

For the determination of natural folates however, enzymatic digestion is required.

The reason for this is two-fold. Firstly, folates can be bound to or trapped within other matrix components such as proteins or carbohydrates. Amylase and protease enzymes are used to break down these compounds to release the folates for analysis. The fat content of foods is not normally a problem as folate does not generally occur in significant amounts in fatty foods however, in some cases the use of a lipase may also be considered.

Secondly, natural folates exist as polyglutamates with varying number of glutamate molecules attached to the pteroyl structure. For microbiological assay, it is necessary to remove the majority of these as the organism response to compounds higher than the diglutamate is significantly reduced. It is possible to separate all of the glutamate forms using HPLC but this can be very complex. Since the glutamate chain length, primarily only affects absorption in the gut, it is usual practise to remove the glutamate side chain during extraction. This is accomplished by use of a γ- glutamyl hydrolyase (deconjugase) enzyme. This enzyme has to be extracted from natural sources such as pigeon liver or rat plasma and its commercial availability is poor. In-house concentration or purification procedures may be required.

In the past, extraction was often carried out using hot, aqueous extraction followed by addition of the deconjugase enzyme only. In the past decade or so however, several workers have shown that a “trienzyme” extraction is required to maximise the folate recovery from food. Sequential or simultaneous extraction with amylase, protease and deconjugase generally results in higher yields of folate from foods, however, the optimal extraction conditions vary from sample to sample and should be verified in the laboratory for the type of samples to be analysed.

1.4.4 Analyte stability

Foods should be analysed as soon as possible and care is needed to ensure that food extracts etc are stored appropriately. Solutions may be kept chilled for a few hours but should be stored in a freezer at -18°C or at -80°C for longer term storage.
It is necessary to add antioxidants during sample extraction to prevent losses of folates. Typically, ascorbic acid is used although the additional use of 2-mercaptoethanol may be desirable to maximise stability.

1.4.5 Removal of Interferences

Folates are present naturally in foods at very low concentrations and it is usually necessary to concentrate them before analysis and to remove other components of the food matrix which would otherwise interfere with the subsequent determination. This can be carried out using a solid phase extraction cartridge in strong anion mode (SAX) although the concentration and

clean-up is very limited using this technique. A preferred approach is to use immobilised folate binding protein to selectively extract folates from food extracts prior to their determination. This technique provides significantly improved clean-up and concentration although, unfortunately these materials are not commercially available. They have to be prepared in-house and have limited useful lifetime which can significantly add to the time and cost of analysis.

1.4.6 Method of detection

The natural, reduced folates can be made to fluoresce naturally by reducing the pH to ~ 2. This provides a sensitive means for their detection. Unfortunately, folic acid and other non or partially reduced species do not fluoresce and have to be detected by UV spectrometry with a consequent drop in sensitivity. An HPLC system equipped with dual detectors is therefore required. Detection using mass spectrometry may solve this problem although this is not very compatible with the buffers used for extraction. Mass spectrometric detection has not been studied in this project.

2. The determination of folates from food

The aim of this project was to obtain an analytical procedure for the determination of natural and added forms of folate in foods. The method which is based on previous studies carried out at LGC and the work published by Konings *et al*. requires extraction of folates using a trienzyme digestion in the presence of ascorbate and mercaptoethanol as antioxidants. The folates are selectively extracted from the digest using an immobilised folate binding protein before analysis by HPLC using dual (UV & fluorescence) detection.

2.1 HPLC

Previous studies had shown that the individual folates can be separated using a reverse-phase column and a gradient, mobile phase system. Dual detection with UV at 290nm for folic acid and fluorescence detection at Ex 300nm, Em 360nm for the natural folates was required. The HPLC column must be stable at the operating pH (2.0) required.

A Genesis C18 (2) column, 3µm, 150 x 4.6mm was selected for the study.

The following conditions were established:

Column:	C18 (2) 150mm x 4.6mm, 3um.
Column temperature:	30°C
Mobile Phase:	A - 30mM Phosphate Buffer, pH 2.0

B - Acetonitrile

Flow rate: 1ml/min

Gradient:

Time	Mobile Phase A	Mobile Phase B
0 min	95%	5%
20 min	80%	20%
28 min	80%	20%
30 min	95%	5%
40 min	95%	5%

Fluorescence detector: Excitation wavelength – 300 nm

Emission wavelength – 360 nm

UV detector: 290nm

Injection volume: 100µL

Run time: 40min

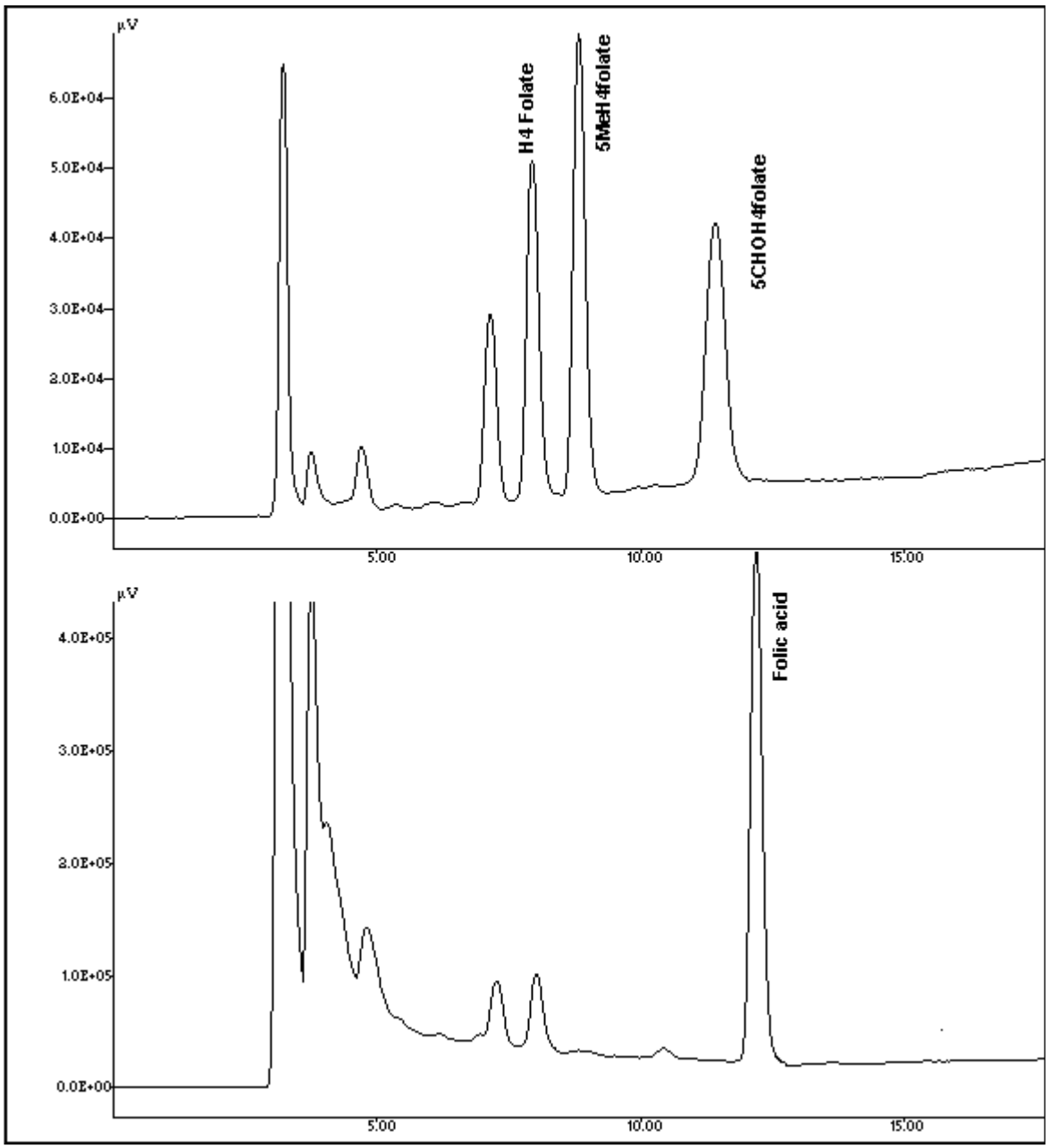

Figure 2: Chromatogram of standards using Fluorescence *(Upper)* and UV *(Lower) detection*

2.2 Target Analytes

The folate compounds most likely to be found in foods are 5-methyl tetrahydrofolate (5MeH_4folate), 5-formyl tetrahydrofolate (5CHOH_4folate) and tetrahydrofolate (H_4folate). Other folates may be found but are not generally significant in relation to overall folate activity. The project focussed on these folates together with folic acid for fortified products.

2.3 Preparation of Standards

5 Me-H_4folate, H_4folate, folyl triglutamate and folyl hexaglutamate were purchased from Schirks Laboratories in Switzerland. Folic Acid and 5CHO H_4folate (folinic acid) were purchased from Sigma-Aldrich. Standards solutions were prepared as follows:

2.3.1 Standard Materials

2.3.1.1 Folic acid monoglutamate (Folic acid) – Sigma, (MW 441.4)

2.3.1.2 5 methyl, tetrahydrofolate (5Me-H_4 folate) – Shirks (CH), Ca salt (MW 497.5)

2.3.1.3 5 formyl tetrahydrofolate (5CHO-H_4 folate; folinic acid) - Sigma or Shirks, Ca salt (MW 511.5)

2.3.1.4 Tetrahydrofolate (H4folate) - Shirks, 3HCl (MW 554.8)

(Note: The purity of these products may vary and their concentration should be verified on use, using UV spectrophotometry.)

2.3.2 Stock Standards

Folate standards are labile to different degrees, especially in the absence of antioxidants and the purity of the available products is highly variable and generally less than 100%. Stock solutions should be stored at -20°C in borate buffer containing 1% ascorbic acid as antioxidant and are stable for at least 3 months under these conditions. Working solutions should be prepared on day of use. Preparation of standards should be carried out so that the minimum possible time elapses before the standard is diluted with the final buffer which contains sodium ascorbate as an antioxidant. Since it is not possible to carry out UV spectrometry on the standard solutions in the presence of high concentrations of ascorbate, two sets of standards are prepared in parallel. The first set is used to determine the standard concentration and are discarded after use. The second set (containing antioxidant) is used to prepare the calibration standards. These standards should be prepared at the same time and subjected to the same processes.

2.3.2.1 UV Concentration check

Each standard was prepared separately. 10mg of standard (corrected for salt content as necessary) was dissolved in 20mL of sodium tetrahydroborate buffer (0.05M) to give stock solutions at 500 µg/mL. 2mL of the stock solution was immediately diluted with phosphate buffer (0.1M, pH7.0) to give a 10µg/mL solution. The absorbance of this solution was used to calculate the standard concentration using the molar absorption coefficients shown in Table 2.

Table 2: Molar Absorption Coefficients of folate compounds

Compound	pH	λ max	Molar Absorption coefficient (ε) (mmol/L/cm)	MW of salt-free form
Folic acid	7	282	27.6	441.4
5Me H_4folate	7	290	31.7	459.4
5CHO H_4folate	7	285	37.2	473.4
H4 folate	7	297	29.1	445.4

Ref: The biochemistry of folic acid and related pteridines, Blakeley, 1969, North Holland publishing.

2.3.2.2 Stock calibration solutions

The stock calibration solutions were prepared in parallel to the UV check solutions but were diluted in phosphate buffer containing 1% sodium ascorbate as antioxidant. These stock solutions were aliquoted into 2mL, capped HPLC vials and were stored at -18°C until needed. (NB -80°C may be preferable if available)

2.3.3 Mixed Intermediate Standard

A mixed intermediate standard was prepared with the following nominal concentrations, which reflects the relative sensitivity of detection for the different folates:

Table 3: Folate concentration of intermediate standard

H_4 Folate	10 ng/mL
5Me H_4 Folate	10 ng/mL
5CHO H_4 Folate	100 ng/mL
Folic acid	1000 ng/mL

One vial of each standard was thawed on the day of use. Appropriate volumes of each standard (diluted additionally with phosphate buffer if required) were pipetted into a 25mL volumetric flask and made to volume with phosphate buffer (pH7.0) containing 1% sodium ascorbate. This standard was stored in a fridge.

2.3.4 Calibration standards

A range of calibration standards was prepared on the day of use. The intermediate standard solution was diluted with phosphate buffer (pH 7.0) according to table 4.

Table 4: Calibration standards

Intermediate Std mL	Final Volume mL	$5MeH_4$ ng/mL	H_4 ng/mL	$5CHOH_4$ ng/mL	PGA ng/mL
10	10	10	10	100	1000
5	10	5	5	50	500
2	10	2	2	20	200
1	10	1	1	10	100
1	20	0.5	0.5	5	50
1	50	0.2	0.2	2	20

NB: These are nominal concentrations. True concentrations should be calculated from the concentration check data.

The intermediate and calibration solutions should be prepared fresh daily and stored in a fridge when not in use.

2.4 HPLC Calibration

The response obtained may vary with the sensitivity of the detectors used and must be established at the point of use. The fluorescence response for $5CHOH_4$folate was weaker (and the peak broader) than that for H_4folate and for $5MeH_4$folate, therefore the detection limit was higher. The detection limit for folic acid using UV detection was also higher. Mixed standards were therefore prepared in the following ratios:

H_4: $5MeH_4$: $5CHOH_4$: Folic acid = 10: 10: 100: 1000

The calibration lines were linear for the following concentration ranges:

5 Me H4 folate	0.2 – 10 ng/mL
H4 folate	0.2- 10 ng/mL
5CHO H4 folate	2 – 100 ng/mL
Folic acid	20 – 1000 ng/mL

Calibration lines for each analyte are shown in Figure 3 overleaf:

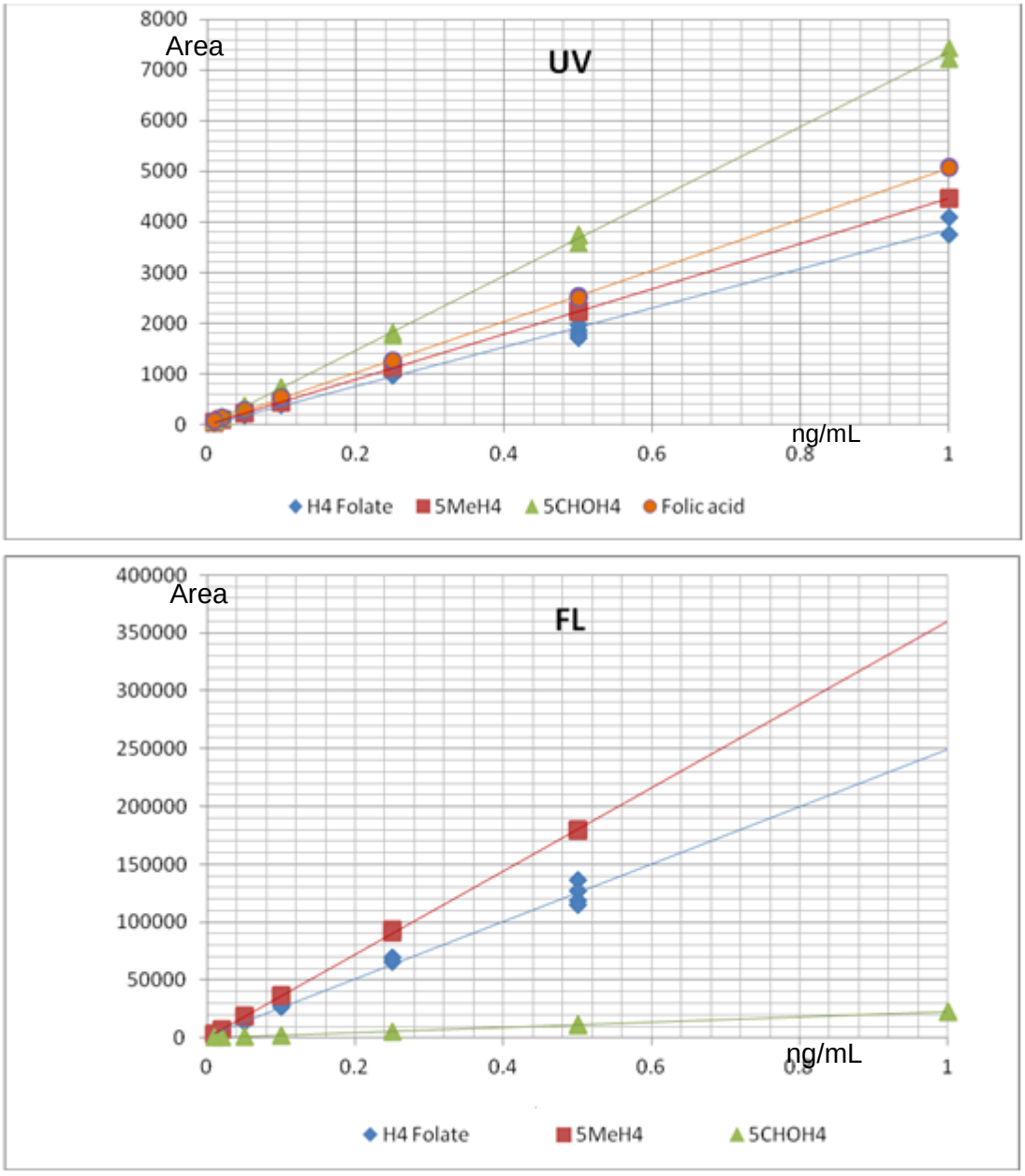

Figure 3: Calibration curves for folates using UV & Fluorescence detectors (µg/mL)

Although it was possible to use the UV detector for all of the folate forms when using standards, in practice, the sensitivity is too low and the chromatograms too noisy to enable its use in samples. Fluorescence detection is preferred for all forms other than folic acid.

3. Preparation of folate binding extraction (affinity) cartridges

In the majority of cases, sample extracts require concentration and removal of interfering components “clean-up” before they can be determined using HPLC. The most effective way to do this is by using a folate-binding material which will selectively extract folates from food. Such a material is not available commercially so must be prepared in-house.
To do this, folate binding protein was purchased from Sigma UK Ltd and was immobilised onto Sepharose gel which was then packed into small cartridges for subsequent use.

Note: Folate binding protein (FBP) can also be extracted from cow’s milk or from pig’s blood. Although FBP obtained in this way may have superior folate retention capability, the additional steps required to do this add to the time and cost for this process. Commercially available FBP was therefore used.

Folate binding cartridges were prepared using the method of Konings *et al*. (*JAOAC INT, Vol 82(1), 1999, pp 119-127*). Folate binding protein (3 mg) was coupled to Affi-Gel 10 resin overnight using the prescribed procedure. The resin was then distributed between six plastic, chromatography cartridges as shown in figure 4.

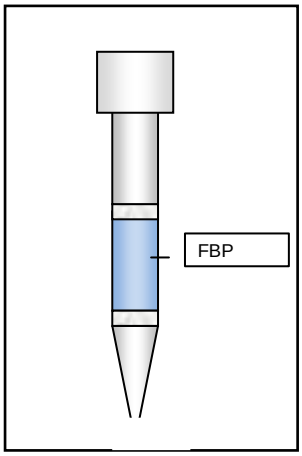

Figure 4: Folate binding affinity cartridge

The gel was rinsed with phosphate buffer and the cartridges were stored in a fridge.

3.1 Folate binding capacity

One mg of folate binding protein is stated by the manufacturer to bind 8µg of folate. In theory therefore, each of the six columns prepared should bind 4 µg of folate. The capacity of the columns to bind folate was tested by overloading the columns with excess folate, washing the

columns and then eluting the retained folate. The amount of folate eluted is equivalent to the maximum that the column can retain under the conditions of use.

The cartridges were loaded with ~ 50 ug of each of the folates separately. After washing, the columns were eluted and the retained folate determined using HPLC. The results are shown below:

Table 5: Folate binding capacities of prepared cartridges

Component	RUN 1	RUN 2
	ug retained	ug retained
H4	4.6	4.5
5Me	4.6	4.6
5CHO	3.2	3.1
Folic acid	5.4	2.2

The loading capacities for H_4 folate and for $5MeH_4$ folate were around 4ug as expected whereas the capacity to bind 5CHO H_4 folate was lower at around 3ug. The capacity for folic acid however was variable at between 2 and 5ug. To ensure that no losses due to overloading occur, the amount of total folate expected in a sample aliquot should be kept to less than 1ug.

NB: The extent to which these capacities change when the columns are reused could not be determined at the outset due to their limited availability. This was monitored during subsequent use by the inclusion of a recovery standard in each analytical batch.

3.2 Recovery of mixed folate standards

A mixed standard was prepared at the concentrations shown in Table 6. 5ml of this standard was loaded onto three of the prepared cartridges. The amount of folates in the eluted solution was determined and was used to calculate the standard recoveries shown. This was repeated three times using the same cartridges.

Table 6: Recovery of folates from a mixed standard

Analyte	Run no.	5MeH4	H_4Fol	5CHOH_4	Folic acid
ng/mL		1.0	1.0	10.0	98.6
Total loaded µg		0.005	0.005	0.05	0.49
1	**A**	124	100	100	134
	B	117	100	100	94
	C	110	99	99	92
2	**A**	114	100	100	118
	B	109	94	94	95
	C	107	84	84	98
3	**A**	112	91	91	139
	B	112	97	97	91
	C	109	97	97	96
Mean		113	96	96	106

The recoveries were all acceptable although the 5MeH_4 folate was slightly high. No losses were observed on re-use and there was no evidence of carry-over or other contamination.

4 Analysis of Samples

A range of samples was analysed using a protocol developed from previous LGC studies and the Konings procedure. Five samples were purchased from a local supermarket. These were prepared as follows:

Malted Yeast extract. Stored at ambient temperature and used “as is”.

Lambs Liver. Blended and frozen at -18°C

Frozen Leaf Spinach: Thawed, homogenised and frozen.

Bran Flakes. Milled and stored at ambient temp. (Fortified)

Dried baker’s yeast. Stored at ambient temperature.

Additionally, the following CRM materials were analysed:

CRM BCR 485 Mixed vegetables

CRM BCR 1949a Infant/adult nutritional formula powder

The expected total folate concentrations in these samples were estimated using the certified values for the CRM materials and published literature values for the retail samples. These are shown in Table 7.

Table 7: Expected folate content of test samples

Sample	Mixed veg. CRM	Milk powder CRM	Lambs liver	Bran cereal	Frozen Spinach	Bovril	Dried yeast
Total folate ug/100g	315	237	230	334	52	1000	4000

1 The Mixed veg. CRM is certified for total folate only and is of natural origin (carrots/tomatoes/sweetcorn). Certified range is (315+/-28 µg/100g)

2 The milk powder CRM is a mixed Infant formula/adult nutritional powder and is certified for folic acid and 5MEH4 folate only. (Folic acid - 229.3 ± 6.2µg/100g; $5MeH_4$ - 8.39 +/- 0.31 µg/100g)

Samples were extracted in phosphate buffer and then subjected to a tri-enzyme hydrolysis with amylase, pancreatin and rat plasma deconjugase. Extracts were cleaned and concentrated using the FBP cartridges before analysis by HPLC.

4.1 Extraction

5g of sample was weighed into a 100mL Duran bottle. 80mL of CHES-HEPES buffer (50mM, pH 7.85) was added and the extract was blended using an Ultra-Turrax blender for 1 min. The bottle was capped and placed into a boiling water bath for 15 min, swirling occasionally. The hot extract was shaken well before cooling in cold water, adjustment to pH 7.0 with 4M HCl and dilution to 100mL with CHES-HEPES buffer.

10mL of the diluted extract was transferred to a 15mL Falcon tube. 2mL of CHES-HEPES buffer was added together with 50µL of alpha amylase (800U/mL) and 50µL of protease (500U/mL). The extract was incubated at 37°C for 1 hour, mixing occasionally. 0.5mL of thawed rat plasma deconjugase (previously prepared according to Annex A) was added and the incubation continued for a further 3 hours, mixing every 30 minutes.

The tube was put into a boiling water bath for 10 min to deactivate the enzymes, and was then cooled immediately in cold water. The extract was diluted to 15mL with CHES-HEPES buffer (pH7), mixed and centrifuged at 5000g and 2-4°C for 20 min. The supernatant was filtered through a 0.45µm syringe filter into a clean tube and stored in a fridge overnight.

4.2 Clean-up / Concentration

The sample extract was cleaned and concentrated using the folate –binding cartridge discussed earlier using the following procedure.

The cartridge was removed from the fridge and allowed to reach ambient temperature.
5mL of “loading” buffer (phosphate buffer; 0.1M, pH 7.0) was passed through the column to remove the storage buffer. The sample extract (2 – 10mL to contain ~ 1µg of total folate) was loaded onto the cartridge and allowed to drain through under gravity. The cartridge was then washed sequentially with 5mL phosphate buffer (0.025M) containing sodium chloride (1M), and 5mL of phosphate buffer (0.025M). The folates were then eluted into a tube using 5mL of trifluoroacetic acid (0.02M) containing dithioerythritol (0.02M). The eluate was collected in a 5mL volumetric flask containing 200µL ascorbic acid solution (25%w/v in water), 40µl potassium hydroxide solution (600mg/mL) and 5µL mercaptoethanol. Elution was allowed to continue under gravity until the 5mL volume was reached. The sample was then transferred to a vial for HPLC.
For subsequent use, the cartridge was rinsed with 5mL of loading buffer. If not reusing the cartridge on the same day, 5mL of loading buffer containing sodium azide (0.2%) was passed through the column and then topped up before capping the tube. The cartridges were stored in the fridge in this storage buffer.

4.3 Sample results:

The measured folate results are shown in table 8.

Table 8: Analytical results for test samples

Sample	**Folate µg/100g**					
	H_4folate	**5MeH_4folate**	**5CHOH_4folate**	**Folic acid**	**Total**	***Total * Expected***
Mixed Veg CRM	2	248	16	0	266	*315*
Milk formula CRM	0	8	0	216	224	*237*
Lambs Liver	21	166	0	0	187	*196*
Bran cereal	0	0	1	345	346	*334*
Frozen Spinach	<1	49	0	0	49	*52*
Malted yeast extract drink	2	23	27	926	978	*1000*
Dried yeast extract	19	3518	28	0	3565	*4000*

* Based on certified value, label claim or food database figures as available.

In general, the results obtained were satisfactory. No folate profile details were available although the forms of folate found were consistent with earlier work *(LGC - unpublished)*. The milk formula powder was certified for folic acid at 229.3 ± 6.2 µg/100g and for 5MeH4 folate at 8.39 ± 0.31 µg/100g and the results obtained was similar to these values. The mixed vegetable CRM was certified for total folate at 315 ± 28 µg/100g therefore the result obtained was slightly low. The total folate results for the remaining samples agreed quite well with the values expected from label or literature sources.

4.4 Affinity cartridge stability

The stored sample extracts were taken through the FBP cartridge extraction on two further occasions, using cartridges that had already been used previously. The results were low and very variable. Standard recoveries from these cartridges were also low and variable. The HPLC calibration lines obtained for standards were consistent with the earlier assays however; it was not possible to obtain reliable data for the sample extracts. It was concluded that although the affinity cartridges performed satisfactorily when re-used with standards and when first used for sample extracts, they cannot be reliably reused for samples.

In previous work, the affinity material was extracted from cow's milk and was packed into a low pressure chromatography column for use in sample clean–up. The column was reusable for samples under the conditions used at that time. Further work would be required to establish whether the poor stability for the affinity cartridges was due to the folate binding protein source, the different physical format used or to other unknown factors.

5 Conclusion

The method evaluated in this study was successful in determining the added and natural folates present in foods using an HPLC determination.

Folates were extracted from foods using hot, aqueous buffer followed by enzymatic hydrolysis (amylase, protease & γ-glutamyl hydrolyase). These extracts have to be cleaned and concentrated before HPLC which was accomplished using non-proprietary affinity cartridges which were prepared in-house. The results obtained for a range of test samples were close to the expected total folate values reported for these samples.

Unfortunately, the affinity cartridges were not reusable for sample extracts and this, combined with the time and effort required to produce these materials, limits the usefulness of this procedure for routine laboratory analysis

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Mention of a commercial analytical product in this report does not imply endorsement by the Government Chemist.

Annex A: Preparation γ-glutamyl hydrolase (deconjugase) from Rat Plasma

100mL of rat plasma (Sprague Dawley, Lithium Heparin, Mixed gender, Pooled) was placed into dialysis tubing (MWCO 12000-14000) and dialysed in 2L of CHES-HEPES buffer (50mM, pH 7.85) containing 2% sodium ascorbate, 0.7mM mercaptoethanol and 4g of activated charcoal. The plasma was dialysed for 24h at 4°C.

The dialysate was aliquoted into 0.5mL eppendorf tubes and stored in a freezer at -80°C.

Activity Check

A standard solution of pteroyl triglutamate (Shirks Labs) in phosphate buffer at pH 7.0 was prepared at a concentration of 200ng/mL.

0.5mL of this standard was placed into two falcon tubes. 0.5mL of rat plasma dialystate was added to one tube only. Both tubes were incubated at 37°C for 30min and then heated to 100°C for 5 minutes. After cooling, the extract volume was made to 10mL with phosphate buffer. Both solutions were injected onto the HPLC system

The peak corresponding to the Pte Glu3 had largely disappeared and that corresponding to folic acid had increased. The recovery compared to the unconverted solution was > 95%. (The theoretical conversion capability is ~ 40nmol (28ug) in 20min at 37°C).