An Evaluation of Procedures for the Determination of Folic acid in Food by HPLC

LGC/R/2011/180

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GC11/0004A

An Evaluation of Procedures for the Determination of Folic acid in Food by HPLC.

A Government Chemist Programme Report

Report number LGC/R/2011/180

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September 2011

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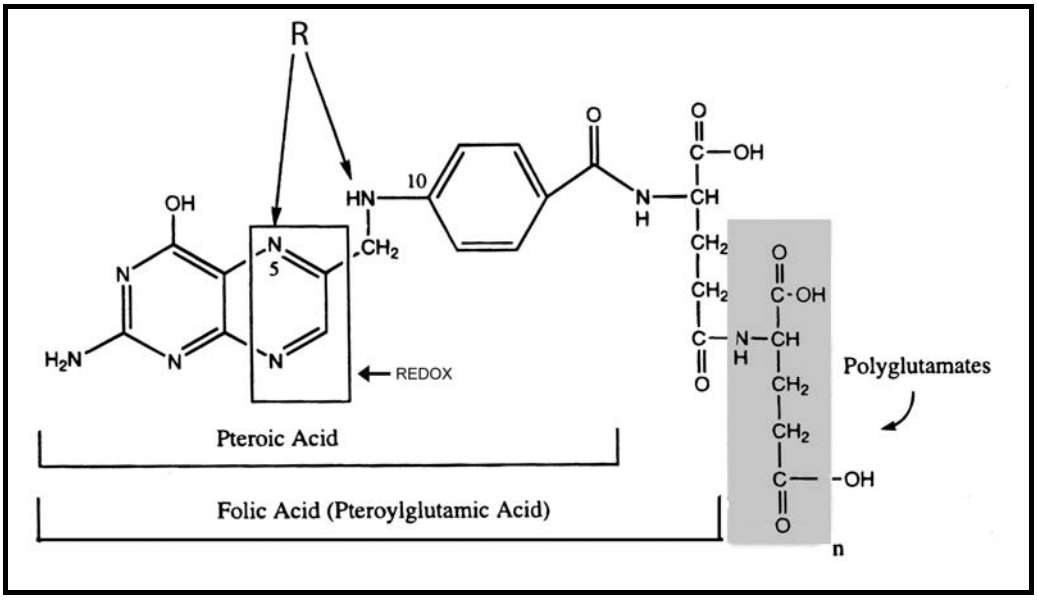
1. Executive Summary

Procedures for the determination of folic acid and folates in foods, supplements and vitamin premixes using High Performance Liquid Chromatography (HPLC) and Ultra-High Performance Liquid Chromatography (UHPLC) were evaluated in this study. The report describes procedures for the determination of added folic acid in vitamin premixes and in infant formula milk. The analysis of natural folates is discussed and a suitable HPLC system has been tested but no samples were analysed. Further work will be carried out in this area. The study was undertaken to underpin the capability of the Government Chemist to perform referee analysis of vitamins by developing appropriate analytical methods.

The report includes an evaluation of a commercial, immunoaffinity cartridge used for the selective isolation of folic acid. The evaluation does not imply any endorsement of the product by LGC or the Government Chemist and alternative products may be available.

2. Background

Folate is a general term for a number of folic acid derivatives which have the biological activity of folic acid (pteroylglutamic acid). These compounds differ by their state of oxidation, the substituents on the pteridine ring, and the number of glutamate residues.



REDOX - This part of the pteridine ring is oxidised (two double bonds) in folic acid but fully reduced in naturally occurring, tetra-hydro (H4) folates.

R = H, methyl, formyl, methylene or methylidine

Figure 1 : Structure of folate compounds

Folates are found naturally in many foods. Good dietary sources of folate include vegetables (especially green vegetables), potatoes, cereals and cereal products, fruits, milk, and organ meats such as liver and kidney. In unfortified foods, folate is present as reduced or partially reduced pteroyl polyglutamates in which the reduced pteroyl compound, usually substituted by methyl or formyl groups at the 5 or 10 positions on the pteridine ring, is attached to a polypeptide chain containing up to 7 glutamic acid units (Fig 1). Folic acid (pteroyl glutamate) does not occur naturally but is used for fortification of foods and supplements.

In vivo, folates are initially deconjugated (glutamate units removed) by γ-glutamyl hydrolase (folyl deconjugase) enzymes in the cells of the intestinal brush-border before absorption. The length of

the glutamate chain primarily affects the time required for absorption of folate in the gut. Once absorbed, folic acid or partially-reduced forms of folate are subsequently reduced to tetrahydrofolate (H_4folate) by the action of folate and dihydrofolate reductase. Further metabolism results in the formation of 5-methyl H_4Folate, 5, 10-methylene H_4folate and methylidyne H_4folate, which are the active metabolites. 5-methyl H_4Folate is the major circulating form in humans. The absorption and rate of conversion to active metabolites is complex and varies depending upon the form of folates present in the foods ingested.

In the laboratory, folates have classically been determined using microbiological assay (MBA) with specific micro-organisms such as *L. Casei Rhamnosus* and this is still in common use. Although the variance of the procedure is comparatively high, this technique gives a similar response for the different forms of folate in foods and therefore gives a reliable estimate of total folate. A major consideration is that the organism will not respond to polypeptide forms with greater than two glutamic residues. Consequently, folates must be deconjugated before determination. It is not possible to get information about the different forms of folate present without complex differential assays.

Methods for the determination of folate by HPLC are becoming more available, and allow differentiation between folate forms, but these still suffer from poor sensitivity, varying response to different folates, interconversion of folate forms during extraction and interference from other food matrices. Whilst separation of the polyglutamate folate forms by HPLC is possible, the resulting chromatograms are complex and difficult to interpret and deconjugation is usually carried out to simplify the determination.

Although these factors are critical when analysing unfortified foods, where fortified foods are concerned, the concentration of folic acid added is likely to be high enough that the contribution from natural folates may not be significant and it may only be necessary to determine the added folic acid. The extraction and HPLC procedures are then much simplified.

3. Objective

The primary aim of this project was to evaluate selected procedures for the determination of added folic acid in foods and vitamin premixes using HPLC. Procedures for the determination of natural folates were considered separately.

4. Extraction of folic acid from foods

Folic acid is water soluble, therefore the free form can be extracted into aqueous solution. Folic acid is stable to 100°C at pH 5.0 to 12.0 when protected from light. It is weakly soluble at pH 2 - 4 but the solubility increases with pH. Folic acid is usually extracted with neutral or mildly alkaline buffers at pHs between 7 and 10. Reducing agents such as ascorbic acid, 2-mercaptoethanol and dithiothretitol protect folates and are often added to the extraction medium. Folic acid is stable in dry products but sample extracts should be protected from strong light, extremes of pH, and oxidising agents.

5. HPLC of Folic acid

Folic acid can be determined using reverse-phase HPLC with C18 columns. A number of suitable systems can be found in the literature. Added folic acid can be determined in supplements or fortified foods by HPLC with UV detection after a neutral or slightly alkaline extraction. Additional clean-up may be required to remove non-folate intereferences and at lower levels of fortification analyte concentration may be required before HPLC. A commercial immunoaffinity cartridge was evaluated for this purpose.

6. Determination of folic acid in vitamin premixes

A method was developed for the determination of vitamin premixes used for the supplementation of cereals and beverages.

6.1 Extraction

Samples were extracted with 0.1M potassium phosphate buffer containing 0.1% sodium ascorbate. The buffer was adjusted to pH 8.5 with 10M sodium hydroxide. Diethylene triamine pentaacetic acid DTPA was added if minerals (especially iron) were present. The samples were shaken, ultrasonicated for 5 minutes and then filtered through a 0.45µn filter before $_{UHPLC}$. Standards were prepared in extraction buffer to contain 5-20 µg/ml of folic acid.

6.2 UHPLC conditions

The samples were analysed on an Agilent 1290, UHPLC system using the following conditions:

Table 1: $_{UHPLC}$ conditions for an analysis of folic acid

Column:	Zorbax RRHD Eclipse Plus C18 (Agilent) 2.1 x 100 mm, 1.8 µm.
Mobile Phase:	**A**: 1-pentane sulphonate (0.1 %w/v) in 0.015M phosphoric acid. **B**: A + Acetonitrile (2:1)
Gradient:	**Minutes** / **%A** / **%B** 0 / 95 / 5 1.6 / 74 / 26 3.5 / 42 / 58
Flow rate:	0.8 mL/min
Column Oven Temp:	30 °C
Detection:	UV(DAD) at 290 nm
Injection volume:	10 µL

6.3 Example chromatograms

Chromatograms of a folic acid standard at 10µg/ml and of a vitamin premix sample are shown below:

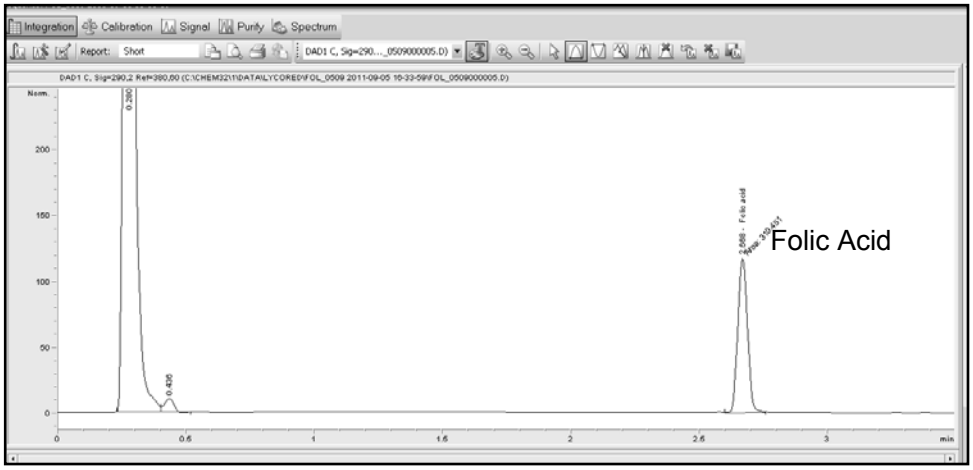
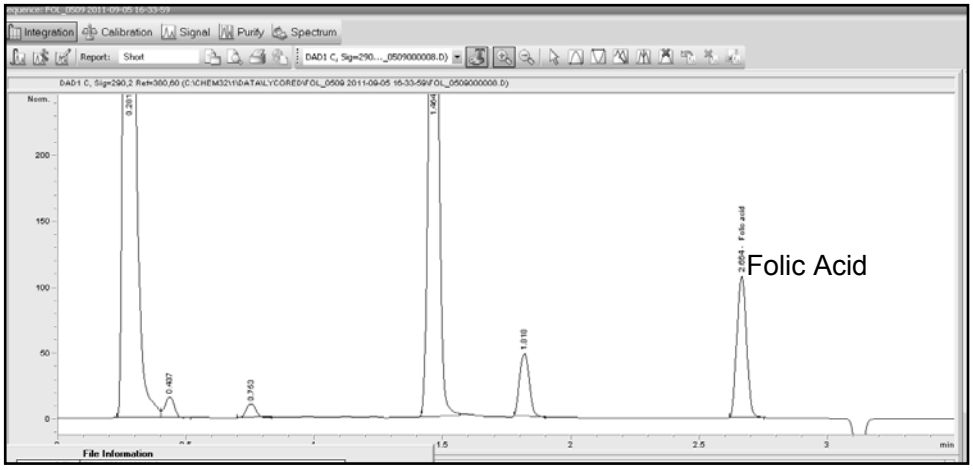


Fig 2: Chromatogram of folic acid standard at 10µg/ml

Fig 3: Chromatogram of a vitamin premix sample



6.4 Calibration

For premixes, the UHPLC system was calibrated using standards containing 5, 10 and 20ug/ml of folic acid. The calibration line is shown below:

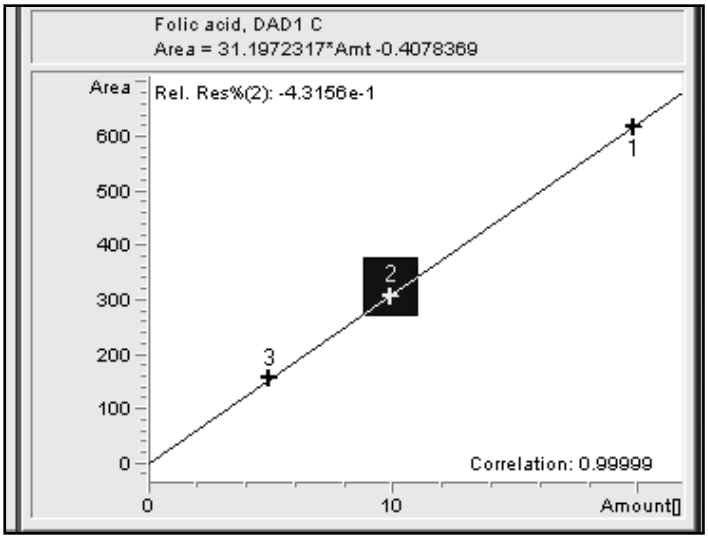


Fig 4: Calibration curve for folic acid

6.5 Analysis of samples

The procedures described above have been used for the analysis of vitamin premix samples with and without minerals, which contain a range of water and oil-soluble vitamins including folic acid.

The folic acid contents for selected samples were as follows:

Table 2: Folic acid results for vitamin premix samples (µg/g)

Sample	Result	Expected Range
A	1063	977 - 1195
B	987	977 - 1195
C	1948	1751 - 2141
D	5448	4995 - 6105
E	107300	100000 - 110500
F	108200	100000 - 110501

All of the results were within the expected ranges and recoveries from spiked samples were > 95%.

7. Analysis of folic acid in Infant formula using a commercial Immunoaffinity cartridge (IAC) and HPLC

An HPLC procedure was used to determine the folic acid content of a liquid infant formula product. The folic acid content of the product was declared on the label as 12µg/100mL.

The sample was analysed using the protocol supplied by the cartridge manufacturer (R-Biopharm Rhone). A 10mL volume of sample was taken to a final extract volume of 100mL. The concentration of folic acid in the sample extract would be too low to measure with the HPLC conditions used, without the use of the IAC cartridge.

7.1 Extraction

10mL of milk was stirred for 5 minutes with 50mL of 0.1M phosphate buffer (pH 7.0) containing 4g of pancreatin and 6mL of 10% sodium ascorbate solution. The extract was then incubated at 37°C for 2 hours. The enzyme was then denatured by heating at 100°C in a steam bath for 20mins and, after cooling, the extract was made up to 100mL with 0.1M buffer. An aliquot of the extract was centrifuged for 10minutes at 4000rpm before filtration using a 0.45µm syringe filter.

(Note: The samples were originally extracted without the use of the pancreatin to remove protein. These extracts were cloudy even after filtration and caused problems with the subsequent IAC procedure. Recoveries of folic acid from these samples were low (~ 60%), therefore it is important to ensure that the sample extracts are clear before proceeding with the IAC step.)

7.2 Concentration and clean-up using an Immunoaffinity cartridge.

The protocol given in the cartridge instructions was followed.

15mL of the sample extract was added to the IAC, followed by a wash with 10mL of water before elution with 1ml of water / acetonitrile (70 / 30) and 1ml of water. The columns were used with gravity elution. A 10 mL plastic reservoir was attached to each cartridge before use. Sample loading should be between 2-3 mL/min to ensure adequate binding but in practice, the drip rate obtained by simple gravity elution was normally sufficient.

An important consideration is that the cartidges have limited capacity to bind folic acid. The maximum capacity is quoted as 0.45 µg of folic acid although the recommended loading amount was 0.1µg. The processed extract will therefore only contain ~0.05 µg/ml of folic acid and a large injection volume is needed for HPLC, which should be calibrated for this concentration. In addition, for samples where the expected folic acid concentration is unknown, care is required when interpreting the analytical results, to ensure that the IAC capacity has not been exceeded as the results for such samples will be under-estimated.

7.3 HPLC

The sample extracts were analysed on a Jasco HPLC system using the conditions given in the cartridge protocol with the exception of the column, and the flow rate. The conditions are shown in the table below:

Table 3: HPLC conditions for analysis of infant milk

<table>
<tr><td>Column:</td><td>Synergy Phenyl Polar RP, 4µm,
150 mm x 4.6 mm i.d.</td></tr>
<tr><td>Mobile Phase:</td><td>A: 0.1% TFA in water
B: Acetonitrile</td></tr>
<tr><td>Gradient:</td><td>
<table>
<tr><th>Minutes</th><th>%A</th><th>%B</th></tr>
<tr><td>0</td><td>88</td><td>12</td></tr>
<tr><td>2</td><td>88</td><td>12</td></tr>
<tr><td>10</td><td>80</td><td>20</td></tr>
<tr><td>15</td><td>80</td><td>20</td></tr>
<tr><td>16</td><td>25</td><td>75</td></tr>
<tr><td>20</td><td>88</td><td>12</td></tr>
<tr><td>35</td><td>88</td><td>12</td></tr>
</table>
</td></tr>
<tr><td>Flow rate:</td><td>0.8 mL/min</td></tr>
<tr><td>Column Oven Temp:</td><td>30 °C</td></tr>
<tr><td>Detection:</td><td>UV(DAD) at 290 nm</td></tr>
<tr><td>Injection volume:</td><td>100 µL</td></tr>
</table>

7.3.1 Calibration

The HPLC system was calibrated over a range from 0.025 – 2 µg/mL. The calibration curve is shown below.

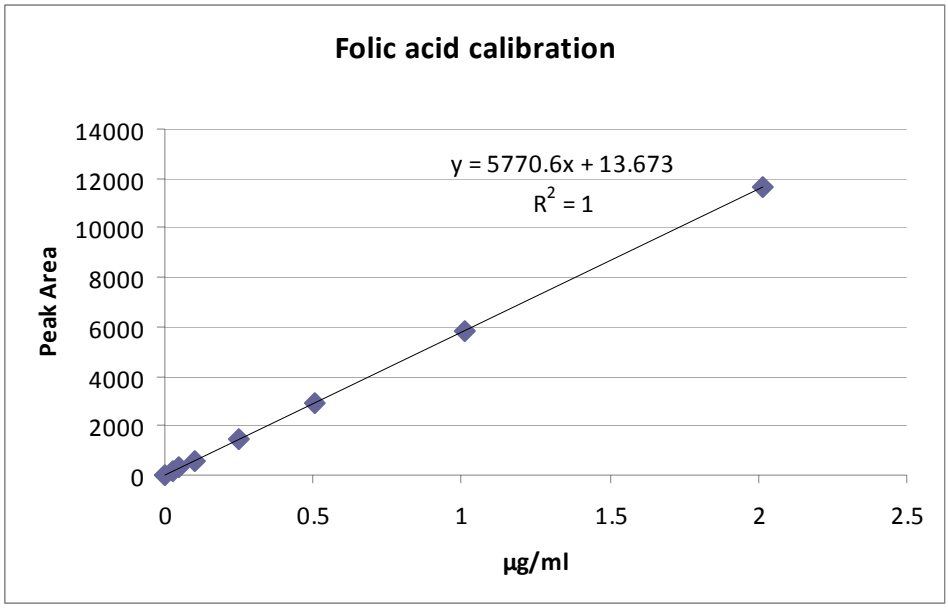


Fig 5: Calibration curve for Folic acid by HPLC

7.3.2 Limit of detection

The limit of detection of the HPLC system as described was approximately 0.01 µg/mL in the injected solution.

7.4 Analytical results

The infant milk was analysed in duplicate using the procedures described above. Two additional samples were spiked with folic acid at ~100% of the original content to assess the analyte recovery. The results and example chromatograms are shown below:

Table 4: Folic acid (µg/100 mL) and recovery (%) results for infant milk

	Sample 1	Sample 2	Mean
Folic acid	11.9	12.4	12.1
Recovery	105.8%	95.5%	100.60%

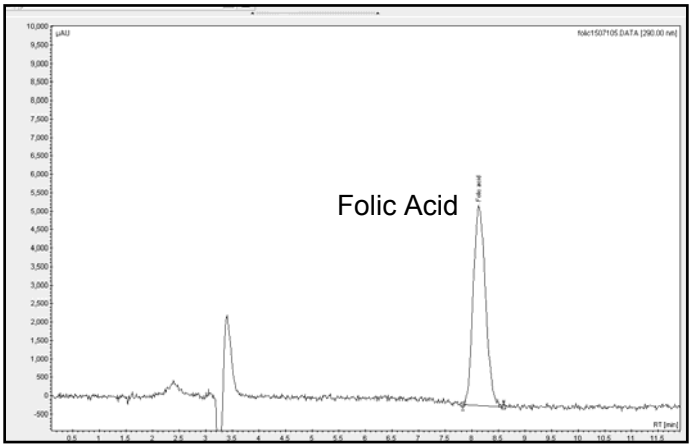


Fig 6: Chromatogram of folic acid standard at 0.25 µg/ml

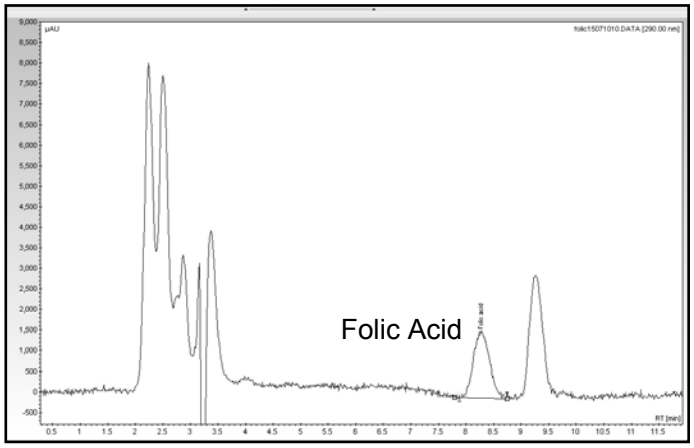


Fig 7: Chromatogram of infant milk sample

7.5 Conclusion

The method prescribed with the IAC is suitable for the determination of folic acid in infant milk. The protocol suggests extraction procedures for a number of other sample types including infant formula powders and cereals. These protocols have not been tested in this evaluation.

8. Analysis of natural folate forms

Folate found naturally in food consists of reduced, substituted forms of which the main ones are 5-methyl tetrahydrofolate (5-MeH_4 folate), 5 (or 10) - formyl H_4folate, and H_4 folate. These may be conjugated to up to seven L-glutamate units. Folic acid does not occur naturally but is used for fortification.

Analysis of the natural folates is complex due to their low concentration in some foods, the presence of other sample components in the food matrix and because of the need to extract and separate different folate forms. Some forms are unstable and may degrade or convert to other forms during extraction and analysis.

Although it is possible to separate folates according to their glutamate chain length, this is very complicated and in practice, this only affects the rate of absorption *in vivo*. Consequently, most analytical procedures include an enzymatic hydrolysis with folyl deconjugase to hydrolyse the glutamate chains so that only mono or diglutamates remain. This deconjugase is not generally available commercially and has to be prepared from one of several substrates such as rat plasma, hog kidney or chicken pancreas. Other enzymes (amylase and protease) are also required to ensure that the folates are fully extracted from the food matrix.

Extracted folates may be determined using microbiological assay if a total folate value is required but HPLC is necessary if information about the forms of folate present is needed. Sample concentration and clean-up is usually required for the determination of folates at natural concentrations. Previous work has shown that although this can be partially achieved using solid – phase cartridges, more specific cleanup using folate-binding protein gives improved results. Unfortunately, this media is not commercially available and must be prepared in-house.

In this project, the aim was to evaluate the commercially available IAC cartridge (see Section 7 above) for the potential to retain natural folate forms and to carry out an initial evaluation of HPLC systems.

8.1 Evaluation of a commercial IAC cartridge

Individual standards of folic acid, H_4folate, 5Me-H_4folate, and 5CHO-H_4 folate were prepared at ~ 0.2 µg/ml in phosphate buffer (pH 7.4). 1ml of each solution was passed through the IAC cartridge which was processed according to the manufacturer's instructions. The processed extracts, (which should contain 0.1µg /ml of folate) were injected onto an HPLC system (see 8.2) and were compared with similar standards that had not been processed using the IAC cartridge.

Only folic acid was recovered from the cartridge procedure whereas the other forms were not retained. This was as expected and confirmed that the antibody used for the IAC cartridge is specific to folic acid only and that it cannot be used to extract natural folates from foods. Further studies on the use of media containing immobilised folate binding protein (which does retain the natural forms) will be carried out in the next GC program.

8.2 HPLC of natural folates

8.2.1 HPLC column

A number of HPLC systems were evaluated for the separation of natural folates in standard solutions. Their performance in separating the folates of interest is summarised in the following table:

Table 5: HPLC column evaluation

Column	Supplier	Dimensions	pH stability	Separation	Peak shape	Retention times	Run time
PLRPS	Polymer Labs	250 x 4.6	Good	Good	Good	Variable	40 min
Phenyl	Waters	75 x 3.9	Poor	Good	Poor	Variable	18 min
Prodigy ODS2	Phenomenex	250 x 4.6	Very good	Good	Good	Constant	39 min *
Genesis C18	Jones	250 x 4.6	Very good	Good	Good	Constant	39 min *
Luna C18 (2)	Phenomenex	250 x 4.6	Very good	Good	Fair	Constant	39 min *

* the last peak eluted at ~ 20 minutes. Runtime includes wash & re-equilibration

All of the above columns were able to separate the folates of interest but the Genesis and Prodigy columns had the best overall performance and stability.

8.2.2 HPLC conditions

The natural folates fluoresce at acid pH therefore a fluorescence detector can be used to enhance the sensitivity and selectivity of the determination. Folic acid however, does not fluoresce and must be determined using a UV detector at 290nm. Dual detectors are therefore required if both natural and added folates are present.

The following system was used:

Table 6: HPLC conditions for natural folates

<table>
<tr><td>Column:</td><td>Genesis C18, 5µm,
250 mm x 4.6 mm i.d.</td></tr>
<tr><td>Mobile Phase:</td><td>A: 30mM phosphate buffer (pH 2.0)
B: Acetonitrile</td></tr>
<tr><td>Gradient:</td><td>
<table>
<tr><th>Minutes</th><th>%A</th><th>%B</th></tr>
<tr><td>0</td><td>95</td><td>5</td></tr>
<tr><td>20</td><td>80</td><td>20</td></tr>
<tr><td>28</td><td>80</td><td>20</td></tr>
<tr><td>30</td><td>95</td><td>5</td></tr>
<tr><td>40</td><td>95</td><td>5</td></tr>
</table>
</td></tr>
<tr><td>Flow rate:</td><td>1.0 mL/min</td></tr>
<tr><td>Column Oven Temp:</td><td>30 °C</td></tr>
<tr><td>Detection:</td><td>UV(DAD) at 290 nm
Fluorescence Ex 300nm; Em 360nm</td></tr>
<tr><td>Injection volume:</td><td>50 µL</td></tr>
</table>

8.2.3 Chromatograms of Standards

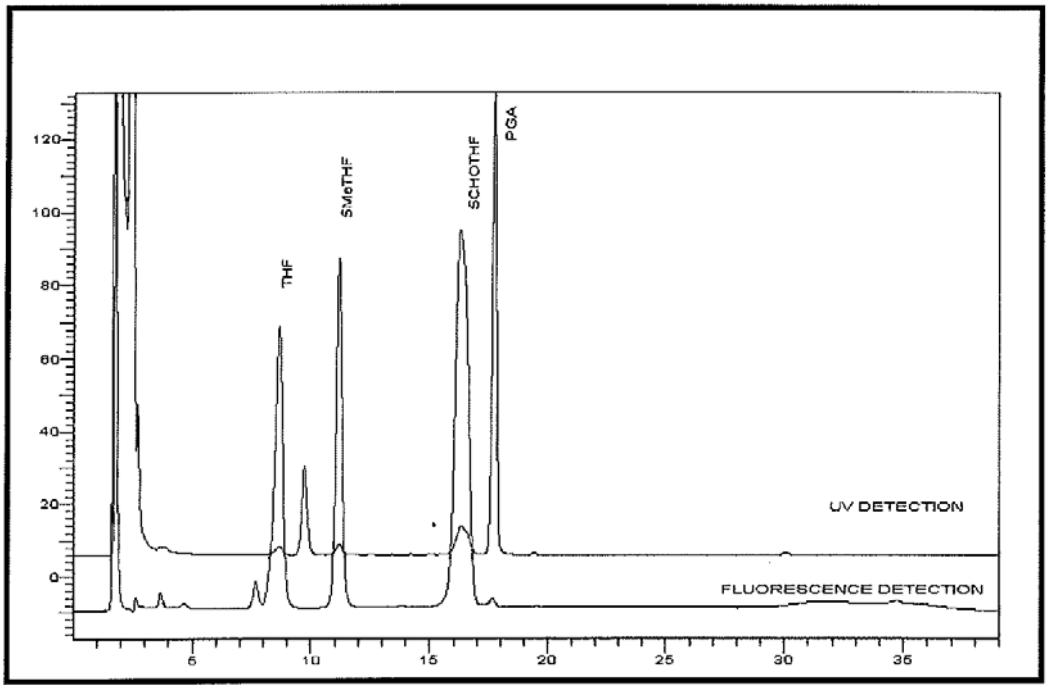


Fig 8: Chromatograms of mixed standards using the conditions described in Table 6

8.2.4 Calibration

Calibration curves are shown below:

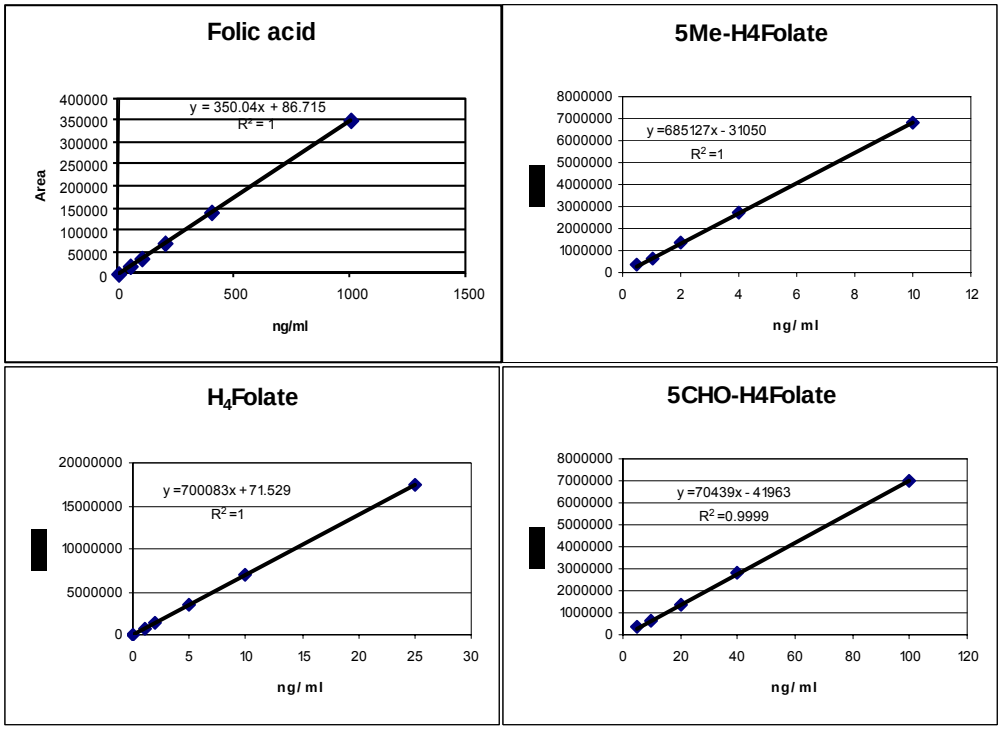


Fig 9: Calibration curves for natural folates

The HPLC conditions described were suitable for the separation of natural folates from food. The chromatograms were linear over the ranges shown. Detection limits vary with the analyte form.

8.2.5 Analysis of samples

No samples were analysed for natural folates in this study.

Until a few years ago, folates were typically extracted by boiling or autoclaving the food in near-neutral or slightly alkaline buffers. The extracted folates were then deconjugated, using a prepared deconjugase, for determination by microbiological assay.

In recent years, “trienzyme” procedures using additional enzymes (amylases and proteases plus deconjugase) during extraction have been published and increased folate concentration has been reported in some foods. It has been concluded that the higher concentrations of folate represent a more complete extraction of the folate from food and therefore provide a more accurate measure of the total folate content.

LGC has considerable experience of the analysis of folates in foods using microbiological assay. Further work will be undertaken in the next programme to evaluate the extraction procedures used and a clean-up / concentration procedure using immobilisd folate-binding protein prior to analysis by HPLC.

9 References

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