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Third EU MAT intercomparison study on food folate analysis using HPLC procedures

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Three samples (milk powder, lyophilized pig's liver and wholemeal flour), a 5-methyltetrahydrofolic acid (5-MTHF) calibrant and two deconjugase enzymes (purified hog kidney and human plasma) were circulated to three laboratories taking part in the study. The objectives were to optimize the deconjugation step in these foods and to improve the between-laboratory agreement in HPLC results for folates. The predominant natural folate form in milk powder was 5-MTHF, together with appreciable amounts of folic acid. In pig's liver 5-MTHF was found to represent about one-third of the total folate content found. For these two foods, results from one laboratory of the sum of the folate vitamers agreed favourably with the microbiological data. 5-MTHF was most successfully determined by all three laboratories. There was little or no agreement found for the other folate vitamers detected. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

This study on HPLC procedures of folate analysis was undertaken as part of the EU Measurement and Testing project on Improvements in Vitamin Analysis in Food. After the first BCR intercomparison study on food folates it was concluded that additional work on the use of HPLC is needed before these assays can confidently be compared to those of the microbiological assay (MA) (Finglas *et al.*, 1993).

The present study was undertaken to overcome some of these difficulties and to improve the reliability and quality of folate data produced using HPLC. The main objectives of the study were to optimize the deconjugation step for use with HPLC methods and to improve the between-laboratory agreement in HPLC results for folates. In addition, information on individual folate forms present in three candidate reference materials (RMs) was needed in order to design protocols for possible certification studies for this vitamin.

MATERIALS AND METHODS

Three samples (a milk powder, lyophilized pig's liver and wholemeal flour) were circulated to three laboratories taking part in the study, and a common 5-methyltetrahydrofolic acid (5-MTHF) calibrant (Schirck's, Switzerland) was also included to check in-house calibration. Two deconjugase enzymes were also circulated: a purified, lyophilized hog kidney enzyme preparation (HK) and a lyophilized human plasma (HP) material (Sigma, P9523). Both enzymes were optimized for use on a yeast powder extract by microbiological assay, and suggested deconjugation procedures for both enzymes were provided.

Each participant was requested to measure total folate and individual folate forms using their in-house procedure. Samples were to be analysed with no deconjugation and after deconjugation using HK and HP enzymes, and an 'in-house' enzyme. The main features of extraction, clean-up and HPLC systems are presented in Table 1.

RESULTS

The predominant natural folate form in milk powder was 5-methyltetrahydrofolic acid, together with appreciable amounts of folic acid (PGA), which was added during preparation of the milk powder. The agreement between laboratories was reasonably good for 5-MTHF

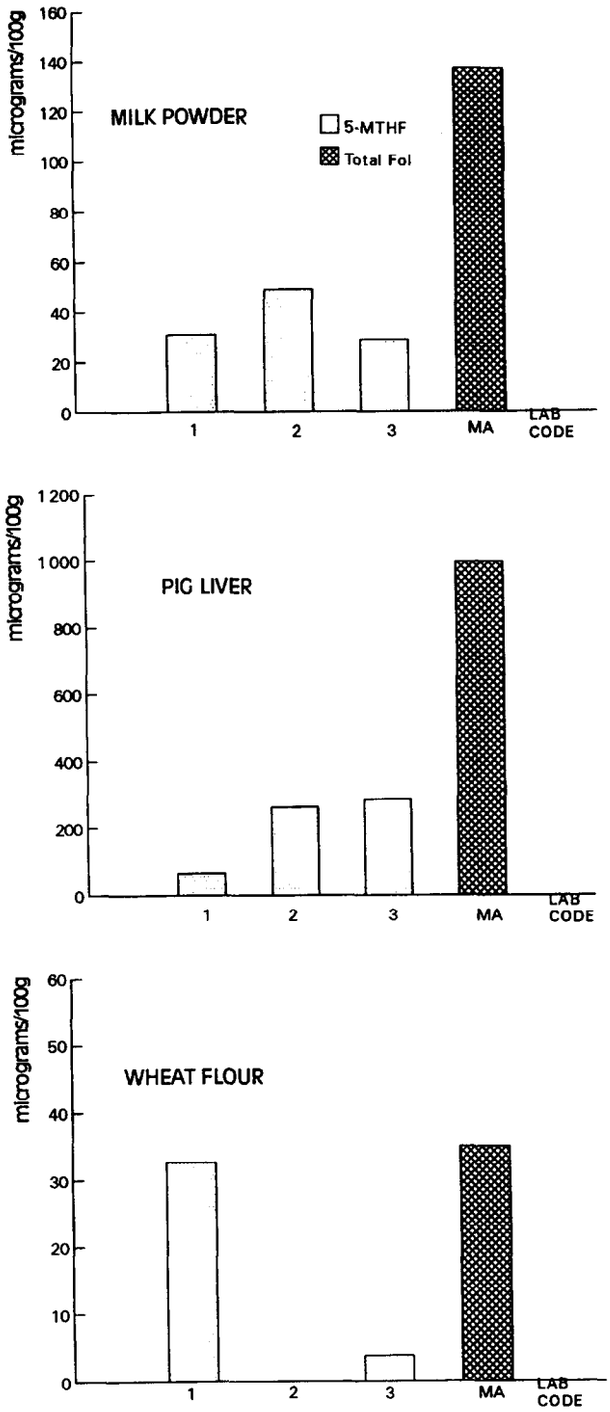

Fig. 1. 5-methyltetrahydrofolate (5-MTHF) in three samples after hog kidney (HK) deconjugation determined by three laboratories using HPLC, and total folates determined using microbiological assay (MA).

given the sparsity of data (Fig. 1). In pig's liver, 5-MTHF was found to represent about one-third of the total folate content found (Fig. 1). For these two foods, results from one laboratory of the sum of the individual folate vitamers agreed favourably with the microbiological data (Fig. 2). Other vitamers detected in these samples included tetrahydrofolic acid (THF), the content of which was high in lyophilized pig's liver,

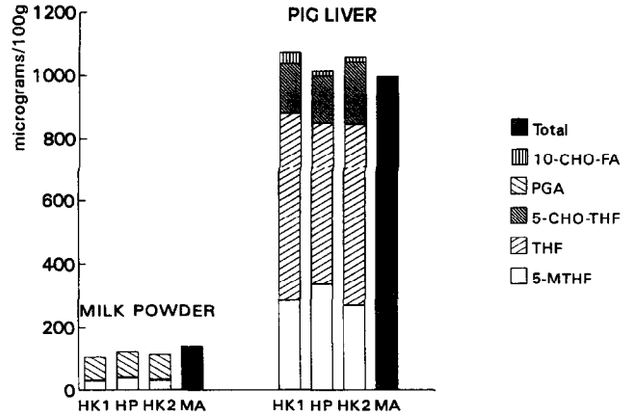

Fig. 2. Individual folate vitamers in milk powder and pig's liver determined by HPLC using three different enzyme treatments, and total folates determined by microbiological assay (MA). HK1, circulated hog kidney conjugase; HK2, in-house hog kidney conjugase; HP, circulated human plasma; MA, microbiological assay; MTHF, 5-methyltetrahydrofolate; THF, tetrahydrofolate; 5-CHO-THF, 5-formyltetrahydrofolate; PGA, pteroylglutamic acid (folic acid) detected at 290 nm; and 10-CHO-FA, 10-formylfolic acid.

5-formyltetrahydrofolic acid (5-CHO-THF) and 10-formylfolic acid (10-CHO-FA). Wholemeal flour proved to be the most difficult matrix to work with, which was possibly due to the low folate levels and the presence of interfering components in the matrix. There was little agreement in the results between laboratories for wholemeal flour (Fig. 1). Deconjugation of 2–3 h was found to be sufficient, although difficulties in the use of HP were reported such as unexpected loss of activity. On the whole, similar results were obtained with each of the enzymes. Increases in the measured folate content after deconjugation was highest in pig's liver, which indicates the highest presence of polyglutamates in this sample.

CONCLUSIONS

In conclusion, 5-MTHF was most successfully determined by all three laboratories in two of the foods, but there is considerable scope for improvements in agreement. For the other folate vitamers, there was little or no agreement found. One possible reason for the variability in results between laboratories was poor stability during extraction unless proper protective measures were taken. Variable deconjugation was sometimes found, although one laboratory reported successful use of all the tested enzymes. At low levels of folates, for example with some vitamers in wholemeal flour, losses during sample clean-up can be high. Poor quality and stability of commercial standards can cause systematic errors in quantitation. Although particular attention was given to peak identification, peak impurities, misidentifications and unsuitable extraction were also among the most probable sources of error. These areas need further investigation before reliable data on individual folates can be obtained.

Table 1. Short description of the analysis method used by each participant

Laboratory	1	2	3
Extraction (buffer, time, temperature)	0.1 M acetate, pH 4.5, 1% ascorbate 15 min, 100°C. For human plasma, treatment samples were extracted in 0.1 M phosphate, pH 6	0.1 M phosphate, pH 6, 1% acetic acid for 10 min in a boiling waterbath	0.075 M phosphate, pH 6, 1% acetic acid–ascorbate, 0.1% mercaptoethanol for 10 min in a boiling waterbath
Deconjugation	2–3 h	3 h	2–3 h
Purification	SAX	SAX	SAX
HPLC column	ODS	ODS	ODS
Mobile phase	acetic acid–ACN (92:8), pH 2.3	5 mM TBAP in 0.1 M H_3PO_4, 11% ACN, 2.5% MeOH, pH 5.1	ACN–30 mM phosphate, pH 2.2, gradient from 9 to 24% ACN in 11 min
Detection	FL (ex 310 nm/em 352 nm)	FL (ex 290 nm/ em 376 nm)	FL (ex 290 nm/em 356 nm) UV 290 nm FL 360/460 nm
Peak identification	spiking	spiking	spiking, dual detection
Reference	modified Gregory *et al.* (1984)	Witthöft & Bitsch (1993)	modified Gregory *et al.* (1984)

SAX, strong anion-exchange solid-phase extraction; ODS, octadecylsilyl-column; TBAP, tetrabutylammoniumphosphate; ACN, acetonitrile; MeOH, methanol; FL, fluorescence; UV, ultraviolet detection; ex/em, excitation and emission wavelengths.

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