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Evaluation of a radioprotein-binding assay (RPBA) for folate analysis in berries and milk

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Abstract

This study aimed to optimise a commercial radioprotein-binding assay (RPBA), routinely used for clinical samples, for folate quantification in foods containing mainly 5-CH_3-H_4folate. The assay was modified using external calibration with 5-CH_3-H_4folate in a lower concentration range diluted in food extraction buffer, rather than the buffer with human serum albumin (HSA) provided by the kit. We evaluated the modified RBPA on some selected food products; milk, whey powder, rose hips, strawberries and European certified reference materials (CRM 421, 485) and the adjustments did not affect the assay negatively. Performance parameters included control of selectivity, absence of matrix effects, recoveries of 94–113%, precision of 1–6 CV% (intra-assay) and 5–9 CV% (inter-assay). Folate concentrations in berries and milk, obtained by the modified RPBA were also compared with other quantification methods such as HPLC and MA. The optimised RPBA was found to be a quick and inexpensive complement to HPLC methods, reliable for folate quantification in foods such as milk and berries that contain mainly 5-CH_3-H_4folate.

Keywords: 5-CH_3-H_4folate; Radioprotein-binding assay; Quality control; Method evaluation; Food analysis; Berries; Milk

1. Introduction

Today the only officially recognised method for folate analysis is AOAC International 992.05. This is a microbiological assay (MA) using *L. rhamnosus* ATCC 7469, and it is only applicable to quantify the "free form of folic acid" (AOAC 992.05, 2000), which restricts its application to fortified products only. Recently, a joint collaborative study with 13 participating laboratories has been carried out quantifying total folate by MA (*L. rhamnosus*) with trienzyme extraction in a wide range of cereal products. This method has now been recommended for Official First Action status with AOAC and First Approval status with AACC (Am Ass Cer Chem) (DeVries, Keagy, Hudson, & Rader, 2001).

High-performance liquid chromatography (HPLC) techniques to separate various folate compounds in food have been more and more established (Finglas, Wigertz, Vahteristo, Witthöft, Southon, & de Froidmont-Görtz, 1999; Konings, 1999; Ndaw, Aoudé-Werner, Lahély, & Hasselmann, 2001; Pfeiffer, Rogers, & Gregory, 1997; Stokes & Webb, 1999; Vahteristo, Finglas, Witthöft, Wigertz, Seale, & de Froidmont-Görtz, 1996; Vahteristo, Ollilainen, Koivistoinen, & Varo, 1996). However, reported HPLC results are contradictory, hence the need for stressing the importance of further method optimisation with respect to peak identification and quantification of native folates other than 5-CH_3-H_4folate (Finglas et al., 1999).

The radioprotein-binding assay (RPBA) based on binding affinity to the folate molecule of a folate binding

Abbreviations: ε, Extinction Coefficient; 5-CH_3-H_4folate, 5-methyl-Tetrahydrofolic acid; 5-CHO-H_4folate, 5-formyl-Tetrahydrofolic acid; Abs, Absorbance; CP, Chicken pancreas; CPM, Counts per minute; CRM, Certified reference material; CV%, Coefficient of variation (%); FBP, Folate binding protein; H_4folate, Tetrahydrofolic acid; HK, Hog kidney; HSA, Human serum albumin; HPLC, High performance liquid chromatography; MA, Microbiological assay; MW, Molecular weight; pABG, Para-aminobenzoyl-L-glutamic acid; Pt-6-COOH, Pterin-6-carboxylic acid; PteGlu, Pteroyl-L-mono-glutamic acid; $PteGlu_2$, Pteroyl-L-di-glutamic acid; $PteGlu_3$, Pteroyl-L-tri-glutamic acid; *R*, Coefficient of correlation; RPBA, Radio protein binding assay; SD, Standard deviation; SPE, Solid phase extraction; wpc 65, Whey protein concentrate (65% protein).

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protein (FBP) derived from bovine milk is today routinely used for clinical samples. A study comparing several commercial RPBA kits for folate analysis of clinical samples showed problems with recovery and variability between kits and stressed the need for further standardisation and optimisation of assay and extraction procedures (van den Berg, Finglas, & Bates, 1994). Attempts to subject the RPBA to folate analyses in foods have revealed several setbacks such as low precision between assays and laboratories, varying affinity to different folate forms and poor agreement with the microbiological assay (De Souza & Eitenmiller, 1990; Finglas, Faure, & Southgate, 1993; Givas & Gutcho, 1975; Gregory, Day, & Ristow, 1982; Shane, Tamura, & Stokstad, 1980; Wigertz & Jägerstad, 1995). Whereas standard calibration and pH are known to be crucial for the RPBA, little attention has been given to disturbances caused by food matrix and buffers. Theobald, Batchelder, and Sturgeon (1981) suggested that matrix effects might change the background radiation and unspecific binding in the assay, resulting in false results. Furthermore, sample preparation in connection to application of RPBA for food analyses and the need of deconjugation using RPBA quantification are still discussed (Finglas et al., 1993; Shane et al., 1980). Shane et al. (1980) observed variable response dependent on the glutamate chain length and considered the RPBA unreliable for the direct determination of polyglutamates.

This study aimed to optimise and validate a commercial RPBA kit for reliable folate quantification in some foods: milk, whey powder, strawberries, rose hips and certified reference materials (CRM 421 and CRM 485). Modifications of the assay included external calibration with 5-CH_3-H_4folate in the concentration range from 0.5 to 10 ng/ml and dilution of standard in the same buffer as the food extracts, rather than the borate buffer with human serum albumin (HSA) provided by the kit. Quality control parameters such as linearity, recovery, precision (intra- and inter-assay), selectivity and reference materials were investigated and the quantified folate content in the foods was compared with other methods such as HPLC (Vahteristo, Ollilainen et al., 1996) and MA using *L. rhamnosus* (AOAC 992.05, 2000). The suitability of some in-house methods for sample pre-treatment, including deconjugation with chicken pancreas or hog kidney conjugases, for RPBA quantification was tested.

2. Materials and methods

2.1. Materials

2.1.1. Solvents and reagents

All reagents were of analytical purity and the water used of Milli-Q grade or equivalent. L(+)-Ascorbic acid (AA), dipotassium hydrogen phosphate, 2-mercaptoethanol (MCE), potassium hydrogen phosphate (K_2HPO_4, KH_2PO_4) and potassium hydroxide (KOH) were purchased from Merck (Darmstadt, Germany). SimulTRAC-SNB Radioassay kit, Vitamin B_{12}[^{57}Co]/Folate[^{125}I] produced by ICN Pharmaceuticals (Orangeburg, USA) was obtained from Boule Nordic AB (Huddinge, Sweden).

2.1.2. Standards

(6-*R*, *S*)-Tetrahydrofolic acid (H_4folate, trihydrochloride salt), (6-*R*, *S*)-5-methyltetrahydrofolic acid (5-CH_3-H_4folate, calcium salt), (6-*R*, *S*)-5-formyltetrahydrofolic acid (5-HCO-H_4folate, calcium salt), pteroyl-L-glutamic acid (PteGlu), pteroyl-di-γ-L-glutamic acid ($PteGlu_2$), pteroyl-tri-γ-L-glutamic acid ($PteGlu_3$), pterin-6-carboxylic acid (Pt-6-COOH) and *para*-aminobenzoyl-L-glutamic acid (pABG) were obtained from Dr. Schircks Laboratories (Jona, Switzerland). Purity of standards was checked as described by van den Berg et al. (1994) using molar extinction coefficients at pH 7 according to Blakley (1969) (Table 1). Standard solutions (200 μg/ml) for calibration purposes were stored at −80 °C in phosphate buffer (0.1 M, pH 6.1), 1.0% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol.

2.1.3. Conjugase (γ-glutamyl hydrolase) sources

Chicken pancreas suspension (CP, 5 mg/ml) was prepared from lyophilised chicken pancreas (Difco, Detroit, USA). CP lyophilisate (50 mg) was ground with 10 drops of glycerol in a mortar. Water (10 ml) was added during stirring and the preparation was centrifuged at 3000 rpm for 5 min. The supernatant was used as freshly prepared. Hog kidney suspension (HK) was prepared according to Gregory, Sartain, and Day (1984) except that lyophilised hog kidney acetone powder from Sigma Co. (St. Louis, USA) dissolved in L-cysteine hydrochloride solution (10 g/l, pH 4.6) was used, rather than fresh hog kidney (Phillips & Wright,

Table 1
Purity control of standard substances (1000 μg/ml)[a] (van den Berg et al. 1994)

Standard substances	λ_{max} (nm)	ϵ[b]	MW (g/mol)	Abs (OD units)	Purity[c] (%)
H_4folate	297	29.1	445.4	0.42	64.9
5-HCO–H_4 folate	285	37.2	473.5	0.55	67.4
5-CH_3–H_4 folate	290	31.7	457.4	0.54	78.2
PteGlu	287	27.6	441.4	0.56	93.5
$PteGlu_2$	–	–	570.5	–	n.d.
$PteGlu_3$	–	–	699.7	–	n.d.

[a] Key: λ_{max} = maximal wavelength, ϵ = extinction coefficient, MW = molecular weight, Abs = measured absorbance at λ_{max}.

[b] Extinction coefficients (OD units/μg/mol/cm) according to Blakley (1969).

[c] Purity = (Abs × MW × dilution factor)/(ϵ × path length). The dilution factor was 100 and the path length 1.0 cm for all substances.

1983). Corrections were made for the folate content of the conjugase suspensions, CP (approx. 15 ng/ml) and HK (< 5 ng/ml).

2.2. Samples

Fresh strawberries (*Fragaria ananassa*, unknown cultivar, 3 kg) were bought at the local market in Uppsala, Sweden. The berries were immediately frozen on trays overnight at −20 °C and stored as subsamples of ca. 100 g in plastic bags until analysis. Prefrozen strawberries of the cultivar *Camorosa* were obtained from ORKLA Foods, Eslöv, Sweden. Prior to analysis, the frozen strawberry samples were cut into pieces within the plastic bag to reduce drip losses. Lyophilised Swedish rose hips (*Rosa Rubignosa*) were also obtained from ORKLA Foods, Eslöv, Sweden. Milk (3% fat) was bought at the local store and stored at −20 °C as subsamples of ca. 50 ml until analysis.

Certified reference materials, CRM 421 (spray-dried milk powder enriched with 0.75 mg folic acid /kg) and CRM 485 (lyophilised mixed vegetables) were purchased from Institute for Reference Materials and Measurements (Geel, Belgium). CRM 421 and CRM 485 were stored as vacuum-packed subsamples of 2–5 g at −80 °C until analysis. A concentrated whey protein powder with 65% protein (wpc 65) supplied by Arla Foods (Götene, Sweden) was used as an in-house reference sample and stored subsampled (5 g) at −20 °C.

2.3. Sample preparation

A flow chart describing the in-house sample preparation, including extraction and enzyme treatment procedures prior to folate quantification in different foods is shown in Fig. 1. Amounts of food sample, buffer and conjugases are given in the figure capture. The extraction buffer was phosphate buffer (0.1 M, pH 6.1), 1.0% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol. To prevent folate oxidation, samples were protected by nitrogen, subdued light and cooled on ice throughout sample preparation. Strawberry samples were homogenised with Ultra Turrax T25 homogeniser (IKA, Germany) before extraction. All samples were flushed with nitrogen, capped and extracted in a boiling water bath for 12 min. The samples were either cooled on ice before centrifugation at 27 000 *g* (Sorvall RC 5B, Du Pont Instruments) for 15 min at 4 °C or subjected to enzyme treatment (see next section). The supernatant was collected, and the residue dissolved with 10 ml of extraction buffer and recentrifuged at 27 000 *g* for 15 min at 4 °C. Supernatants from both centrifugations were pooled and made to volume with extraction buffer. For milk samples, only one centrifugation at 27 000 *g* was carried out.

Conjugases were added to the food samples as shown in the flow chart (Fig. 1). The samples were flushed with nitrogen, capped and incubated in shaking water bath at 37 °C for 3 h. Enzymes were inactivated by treatment in a boiling water bath for 5 min. After cooling on ice, samples were centrifuged and the supernatants portioned into 1.5-ml volumes and stored at −20 °C until folate quantification. When calculating folate concentrations in food, corrections were made for the folate content in the conjugase suspensions added to the food sample during sample preparation.

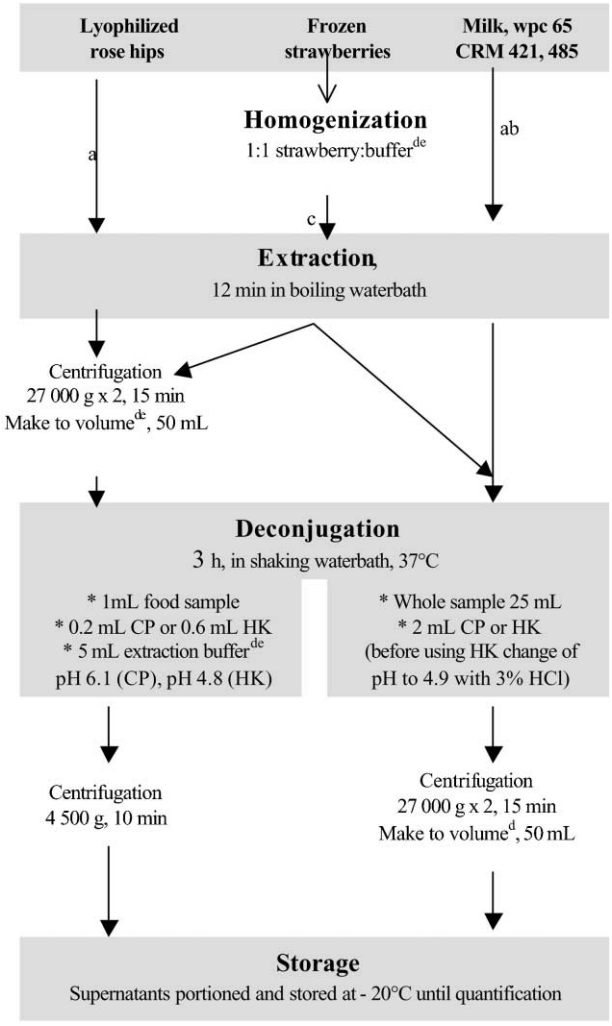

Fig. 1. Flow chart of sample pre-treatment prior to folate RPBA quantification in foods. To prevent folate oxidation, samples were protected by nitrogen atmosphere, subdued light and cooled on ice throughout sample preparation. (a) 0.2–0.5 g of lyophilised food sample and 20 ml buffer; (b) 10 g of dairy products and 20 ml buffer; (c) 4 g of strawberry homogenate and 16 ml buffer; (d) Phosphate buffer (0.1 M, pH 6.1), 1.0% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol; (e) Phosphate buffer (0.1 M, pH 4.8), 1.0% (w/v) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol.

2.4. RPBA

Total folate content of food extracts was determined by a modified commercial RPBA kit, SimulTRAC-SNB Radioassay kit, Vitamin B_{12}[^{57}Co]/Folate[^{125}I] from

ICN Pharmaceuticals (Orangeburg, USA). Food extracts were quantified according to the kit descriptions with some modifications. External calibration with 5-CH_3-H_4folate in the concentration range 0.5–10 ng/ml was used, rather than the kit calibrant (PteGlu) at concentrations from 1 to 20 ng/ml. The external standard was diluted in 0.1 M phosphate buffer pH 6.1 containing 1% (w/v) ascorbic acid, the same buffer as the food extracts, rather than the borate buffer with HSA provided by the kit.

The assay started with addition of aliquots of standard or sample and tracer solutions to tubes in duplicates. Before addition of folate binder in a borate buffer or blank solution (pH 9.5), proteins were denaturated by addition of an extracting reagent containing NaOH, pH (12–13). After a centrifugation, relative radioactivity in pellets was measured by a Cobra Auto Gamma scintillation counter from Packard-Canberra Company (Meriden, USA) linked to the computer software, MultiCalc Routine from Wallac (Turku, Finland).

Logit/log transformation, plotting of standard curve and calculation of folate concentrations in the samples were performed automatically by the Multicalc Routine software. A 50% reduction of maximum binding of tracer in the assay occurred at 2 ng/ml and indicated the middle of the standard curve. Therefore, all food samples were diluted in the same buffer as the 5-CH_3-H_4folate standard to a concentration of approximately 2 ng/ml. The software Multicalc Routine calculated for each assay a precision file indicating the coefficient of variation (CV%) for each point of the standard curve. The correlation coefficient $R > 0.995$ was used as an in-house limit for correlation of the logit/log standard curve between the calibrant (5-CH_3-H_4folate) and the measured radioactivity (CPM). The assay was re-analysed if the back calculated folate concentrations for each calibration point in the standard curve did not fall within $\pm 15\%$. Each sample was analysed in duplicate in the RPBA. An in-house limit for variation between the duplicates was set to 10%, otherwise both samples were re-analysed.

2.5. HPLC analysis and MA

Total folate content of the food extracts was also analysed by HPLC and MA. All samples were treated according to our in-house method previously described in Section 2.3, including HK conjugase treatment for HPLC quantification and CP conjugase treatment for the RPBA and MA. The same extracts in duplicate were quantified when comparing RPBA and MA. The MA was carried out by the Swedish National Food Administration, accredited for folate analysis in foods, using the AOAC Official method 992.05 (2000) with *L. rhamnosus*. Folate quantification by HPLC was carried out as described by Vahteristo, Ollilainen et al. (1996) with some modifications. The analytical column was a Zorbax C8, 150×4.6, 5 μm and the mobile phase consisted of 0.03 M phosphate buffer, pH 2.3, using a gradient with acetonitrile starting at 6% with a lag time of 5 min, raising linearly to 25% within 20 min at a flow of 0.4 ml/min. Individual folates in the samples were quantified with fluorescence detection (ex/em = 290/360 nm). Prior to HPLC quantification, food extracts were purified with strong-anion-exchange solid phase extraction according to Vahteristo, Ollilainen et al. (1996) with minor modifications. 1 ml of extract was applied to the cartridges (Solvent, 200 mg) and eluted with 2.5 ml 0.1 M sodium acetate containing 10% (w/w) sodium chloride, 1% (w/w) ascorbic acid and 0.1% (v/v) 2-mercaptoethanol.

2.6. Internal method evaluation

2.6.1. Recovery

Milk, strawberries, rose hips and CRMs were used in recovery studies with 5-CH_3-H_4folate. To check folate losses during the complete procedures of sample preparation and RPBA quantification, known amounts of the calibrated 5-CH_3-H_4folate standard were added to samples before extraction in concentrations of 50–100% of native folate content.

2.6.2. Selectivity

The binding affinity of the folate binding protein in the kit at pH 9.5 was controlled for six folate derivatives (PteGlu, $PteGlu_2$, $PteGlu_3$, 5-CH_3-H_4folate, H_4folate and 5-HCO-H_4folate) and the kit PteGlu. All standards were diluted in a concentration interval from 0.5 to 10 ng/ml in 0.1 M phosphate buffer pH 6.1 containing 1% (w/v) ascorbic acid, except for the kit PteGlu. Kit PteGlu standards were delivered in a borate buffer with HSA in the concentration range 1–20 ng/ml. Folate quantification was performed as described previously (Section 2.4) and total folate concentrations were compared to the theoretical concentration of 5-CH_3-H_4folate on a molar basis. Moreover, binding affinity of the folate binder to degraded folate products such as Pt-6-COOH and pABG was investigated by quantification of these substances treated as food samples in the RPBA-assay (Section 2.4).

2.6.3. Matrix effects

To check for absence of food matrix effects during RPBA-quantification, food extracts were diluted in the concentration range of the 5-CH_3-H_4folate standard curve (0.5–5 ng/ml). For each food matrix, the effect on pH in the assay was also checked. The pH was measured after addition of folate binder and the last 60 min incubation. Exclusion of the kit's folate binder during the assay and treatment of these samples similar to blank samples allowed control of background radiation. Unspecific background binding was investigated for

several buffers with and without 2-mercaptoethanol and ascorbic acid, and for food extracts of strawberry, rose hips and milk.

2.6.4. Precision

Intra-assay and inter-assay variation during quantification of total folate content with the RPBA-assay was investigated using CRM 485, wpc 65 and strawberry samples. To check intra-assay variation, three to five samples were prepared on the same day and analysed in the same RPBA run. To check inter-assay variation, duplicates of samples were prepared at three to seven individual occasions and quantified in separate RPBA assays.

2.7. External evaluation

Selected food samples were analysed using three different methods of quantification: the optimised RPBA, HPLC (Vahteristo, Ollilainen et al., 1996) and microbiological assay (AOAC 992.05, 2000, *L. rhamnosus*). Certified reference materials, CRM 485 and CRM 421 were analysed according to the flow chart of sample preparation (Fig. 1).

2.8. Statistical analysis

Average concentrations, standard deviations (SD) and coefficients of variation (CV%) were calculated for all samples, generally triplicates, using the computer program Excel 97 from Microsoft. Distribution ranges are given for duplicate samples. To check significant variations between samples and different RPBA standards curves, one-way analysis of variance with general linear modelling by the computer program Minitab Statistical Software release 12, Minitab Inc, was used. Variations from $P < 0.05$ were considered significant.

3. Results and discussion

3.1. Calibration of the modified RPBA

The adjustments with external calibration and buffer in a lower concentration range than the original kit did not affect the assay negatively. The mean slope of calibration curves from 30 individual occasions was 1.09 ± 0.05, the intercept 0.67 ± 0.18, and the correlation of variation $R > 0.995$. CV% for three different calibration points was 13% at 0.5 ng/ml, 5.2% at 2 ng/ml and 9.3% at 10 ng/ml ($n = 30$), respectively. The amount of folate causing a 50% reduction of maximum binding of tracer was 1.85 ± 0.18 ng/ml (50% tracer and 50% unknown sample is bound to the folate binder). This showed the most reliable part in the calibration range to be about 2 ng/ml and therefore, all samples were diluted to concentrations of approximately 2 ng/ml. According to the kit instructions the detection limit is defined as the folate concentration at 90% tracer binding and is reported to 0.6 ng/ml (ICN, 1998). For our external calibration with 5-CH_3-H_4folate the detection limit was 0.2 ng/ml. The kit manufacturer does not report any correlation coefficient, slope or intercept but the folate concentration at 50% reduction of maximum binding is approximately 5 ng/ml (ICN, 1998).

3.2. Sample preparation

To improve folate extraction from the food matrix, the optimal food to buffer ratio during extraction was evaluated and the result corresponds to given amounts in Fig. 1. Recalculated in terms of dry matter, a ratio of 0.2–0.5 g of food sample (dry matter) to approximately 20 ml extraction buffer can be recommended for all foods tested. As outlined in Fig. 1, enzyme treatment was performed in two different ways for strawberry samples, with or without the food matrix remaining in the tubes during incubation. Both procedures gave similar results, indicating a complete extraction of folates from the food matrix prior to enzyme treatment (61.4 ± 1.2 and 60.9 ± 2.4 μg/100 g ($n = 4$), respectively). However, sample treatment with centrifugation before enzyme treatment was routinely used throughout the study. For milk, an extraction in boiling waterbath for 12 min (5.1 μg/100g) was as efficient as autoclaving (4.9 μg/100g). Therefore, an extraction time of 12 min was used for all food matrixes.

No significant variation in folate concentration was found using different in-house methods of conjugase treatments for all tested matrixes, except milk products for which CP conjugase treatment resulted in higher values than HK conjugase treatment or no deconjugation (Table 2). Using different conjugase sources in combination with RPBA quantification, we compared our results with both MA analyses after CP conjugase

Table 2
Effect of deconjugation treatment on folate content in food samples and CRM materials

Food matrix	Folate concentration[a] (μg/100g)		
	CP	HK	No deconjugation
Strawberries[b]	63 ± 3	56 ± 1	54 ± 4
Rose hips[c]	259 ± 7	235 ± 10	235 ± 4
Milk	3.8 ± 0.3	2.4 ± 0.3	3.0 ± 0.3
CRM 421	85 ± 3	69 ± 3	80[d]
CRM 485	223 ± 36	196 ± 27	199 ± 2

[a] Presented results are mean values of triplicates ± standard deviation. Samples extracted as triplicates with CP or HK conjugase and quantified in the same RPBA assay.
[b] Strawberries of the cultivar "Camorosa".
[c] Lyophilised rose hips.
[d] Single value.

treatment and HPLC analyses after HK conjugase treatment. (Quantification by HPLC required deconjugation of folates to monoglutamate form and therefore, a treatment with HK conjugase was necessary.) However, to simplify sample preparation routinely, CP conjugase treatment at pH 6.1 was used throughout this study. Earlier studies have shown that CP conjugase itself possesses significant amylolytic activity and it has also been suggested to have residual proteolytic activity (De Souza & Eitenmiller, 1990; Pedersen, 1988). This could explain the discrepancy between folate concentrations after CP- or HK conjugase treatment for tested milk products in this study. Others have recommended α-amylase treatment followed by protease and conjugase for milk products to achieve maximum yield of folate (Konings, 1999; Lim, Mackey, Tamura, Wong, & Picciano, 1998; Martin, Landen, & Soliman, 1990). Even for other food products, tri-enzyme treatment has become more common and many workers have reported an increase of measurable folate levels in various foods (Konings, 1999; Lim et al., 1998; Pfeiffer et al., 1997; Rader, Weaver, & Angyal, 1998; Shrestha, Arcot, & Paterson, 2000; Tamura, Mizuno, Johnston, & Jacob, 1997). However, these studies are not all in agreement. Furthermore the study by Ndaw et al. (2001) shows that treatment with protease and amylase does not affect folate concentrations in five tested foods (peas, spinach, beef liver, beef fillet, and yeast) compared with only CP conjugase.

Based on our results, we cannot recommend the use of a specific enzyme treatment. It is important to carefully evaluate the use of different enzyme treatments for every new food matrix and tri-enzyme treatment should be considered. This study did not answer the question whether an enzyme treatment is necessary when using RPBA for folate analysis in foods. However, based on the observation by Shane et al. (1980) that the RPBA shows variable response dependent on the length of the folate glutamate chains, we will recommend an enzyme treatment.

3.3. Internal method evaluation

3.3.1. Recovery

In Table 3, folate losses during sample pre-treatment from recovery studies with 5-CH_3-H_4folate are presented. Mean recoveries ranged from 94 to 113% for all food samples and CRMs tested. In the kit description, a recovery test of serum samples is presented, resulting in a recovery range of 98–107% using 5-CH_3-H_4folate (ICN, 1998). Folate analysis in serum requires no sample pre-treatment such as extraction and deconjugation (ICN, 1998). In spite of this, food samples that require extensive sample pre-treatment had recovery results in the same range as for plasma samples, proving folate stability throughout the whole sample preparation.

Table 3
Recoveries of 5-CH_3–H_4 folate added to food samples and CRM materials[a]

Food sample	Amount in food μg/100 g food	Amount added[b] μg/100 g food	Recovery[c] %
Strawberry[d]	63±5	27	102±3
Milk	5±0.3	5	97±7
Rose hip (lyophilised)	259±7	164	103 (92–113)[e]
CRM 421 (milkpowder)	91±5	100	94±2
CRM 485 (mixed vegetables)	204±15	164	113±2

[a] Recoveries are means of triplicates±standard deviation. Samples extracted as triplicates with CP conjugase and quantified in the same RPBA assay.
[b] Amount 5-CH_3–H_4 folate added to the food sample prior to extraction.
[c] Recovery = $(C_{\text{spiked sample}} - C_{\text{sample}}) / C_{\text{spike}} \times 100$.
[d] Strawberries of the cultivar "Camorosa".
[e] Recovery is a mean of duplicate (range).

3.3.2. Selectivity

The response of the modified RPBA to the external standard substances (PteGlu, $PteGlu_2$, $PteGlu_3$, 5-CH_3-H_4 folate, H_4folate, 5-HCO-H_4folate) and the kit PteGlu at pH 9.5 are presented in Fig. 2. The affinity of the FBP to different folate forms varies. The curves can be interpreted as a significant overestimation for H_4folate ($P<0.001$) and significant underestimation for external PteGlu ($P=0.023$), $PteGlu_2$ ($P<0.001$), $PteGlu_3$ ($P<0.001$) and internal kit PteGlu ($P<0.001$). Almost no response was shown for 5-HCO-H_4folate.

In this assay, performed at pH 9.5 the responses of external PteGlu was significantly lower than 5-CH_3-H_4folate (approx. 15%). It has earlier been shown that pH 9.3 is optimal for equal binding of PteGlu and 5-CH_3-H_4folate and that at pH above 9.4, dose–response curves begin to diverge with 5-CH_3-H_4 folate displaying the greater affinity (Givas & Gutcho, 1975). A decrease of pH in the RPBA assay from 9.5 to 9.3 could probably

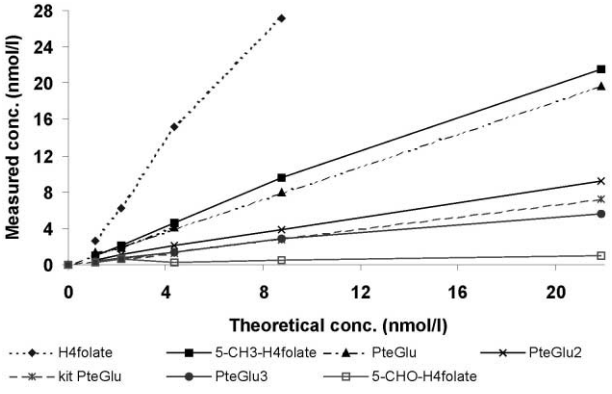

Fig. 2. RPBA response to H_4 folate, 5-CH_3–H_4 folate, 5-HCO–H_4 folate, PteGlu, $PteGlu_2$, $PteGlu_3$ and kit PteGlu at optimal pH of 9.5. Concentrations are calculated against theoretical 5-CH_3-H_4 folate concentrations.

synchronise the responses of external PteGlu and 5-CH_3-H_4folate and increase the performance of the kit.

The response of purity-controlled external PteGlu was much higher than for the internal kit PteGlu (Fig. 2). This is probably because the RPBA kit was originally developed for folate and vitamin B_{12} analysis in clinical samples. The PteGlu standards provided with the kit are therefore diluted in a borate buffer containing HSA to simulate human plasma. Although a denaturation step is included prior to quantification, unspecific binding of the tracer may be caused by HSA. An explanation for the disagreement between standards could also be that the actual concentration of the kit PteGlu was not controlled. This problem was discussed by van den Berg et al. (1994) who reported about a kit manufacturer who supplied standards with assigned values related to a MA, rather than to a primary standard. Regarding the kit used in this study no information is available. Therefore, we strongly recommend external calibration with purity controlled standards, diluted in the same buffer as the food extracts, not the standards supplied with the RPBA-kit, when analysing folates in foods.

The reason for the low response of $PteGlu_2$ and $PteGlu_3$-standards still needs further investigation. The lack of extinction coefficients makes it impossible to solve the problem within this study. Therefore, we cannot comment on the statement by Shane et al. (1980) that varied length of folate glutamate chains result in different response in the RPBA.

However, it is important to be aware of the variable responses from the different monoglutamate folate forms. The affinity for H_4folate is much stronger than for 5-CH_3-H_4folate and there is almost no affinity for 5-HCO-H_4folate. This was also observed by Wigertz and Jägerstad, (1995) and clearly demonstrates the necessity to have knowledge about the dominant folate form within the food matrix to choose a suitable calibrant. This is a restriction: this optimised RPBA with external calibration with 5-CH_3-H_4folate can only be recommended for folate quantification in foods containing mainly 5-CH_3-H_4folate.

No measurable response was found in the RPBA assay for two typical folate degradation products, Pt-6-COOH and pABG. This result indicated the assay to be selective to only biologically active folate forms and therefore suitable to use when studying folate retention in processed food products, where degradation products from oxidised folates could occur and interfere with quantification.

3.3.3. Matrix effects

No effect on the RPBA response, in the calibration range between 0.5 and 5 ng/ml 5-CH_3-H_4folate, was shown by dilution curves of tested food matrixes (Fig. 3). Also, the food extracts did not affect the assay pH showing a pH range between 9.36 and 9.47.

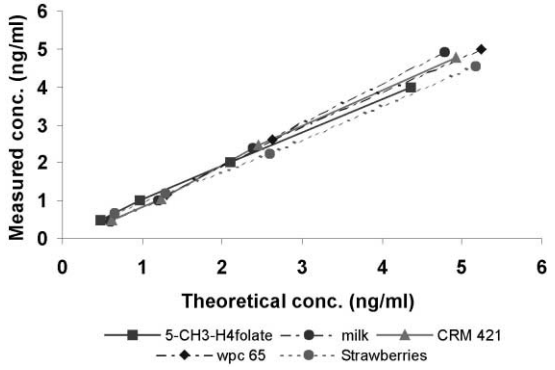

Fig. 3. RPBA response to dilutions of several foods extracts in the range of 0.5–5 ng/ml folate. Concentrations are calculated against theoretical 5-CH_3–H_4 folate concentrations.

Although, as concluded in the Section 3.1, we will recommend that all food samples are diluted to approximately 2 ng/ml folate prior to quantification to standardise the assay.

The background radiation without addition of folate binder remained constant and independent of buffer concentration, reducing agents and absence or presence of food matrix within the range of 94–107% (Table 4). In contrast, the background radiation of the original kit buffer with HSA and plasma samples was 25% higher than that of 0.1M phosphate buffer pH 6.1 used for the optimised RPBA.

Table 4
Effect of molarity, pH and food matrix on the background radiation[a]

RPBA samples	Background radiation[b]	
	CPM	%
Modified RPBA buffer (phosphate 0.1M, 1% AA, pH 6.1)	813	100[c]
Phosphate buffers		
50 mM, 1% AA, pH 6.1	870	107
25 mM, 1% AA, pH 4.9	842	104
25 mM, 1% AA, 0.1% MCE, pH 4.9	805	99
HSA samples		
Kit buffer with HSA, pH 7.8	993	122
Blood plasma sample	1028	126
Food extracts, pH 6.1		
Strawberries, extraction buffer 1, pH 6.1	764	94
Milk, extraction buffer 1, pH 6.1	847	104

[a] Key: AA = ascorbic acid, MCE = 2-mercaptoethanol, HSA = human serum albumin, CPM = counts per minute. All results from one RPBA assay analysed in duplicates. Food extracts were treated as described in the section quantification of material and methods, diluted to 2 ng/ml in the modified RPBA buffer.

[b] Background radiation = CPM value when no folate binder is present in the sample.

[c] Modified RPBA buffer was set to 100%.

We also found a difference in unspecific binding of the folate binder for the 0.1 M phosphate buffer and original kit buffer. The binding of tracer PteGlu was 15% higher for 0.1 M phosphate buffer than the original kit buffer, corresponding to a 15% higher unspecific binding for the original kit buffer. This explains why the RPBA without adjustments and external standard may result in misleading values for folate concentrations in foods and emphasises the need to use the same buffer for standards and samples. This important aspect has been overlooked in previous studies using commercial RPBA kits for analyses of food folates (Andersson & Öste, 1992; Finglas et al., 1993; Wigertz & Jägerstad, 1995).

3.3.4. Precision

The precision of the modified RPBA method including sample pre-treatment resulted in coefficients of variation between 1 and 6% (intra-assay) and between 5 and 9% (inter-assay) for CRM 485, wpc 65 and strawberry samples (Table 5). According to the kit manufacturer ICN (1998), folate quantification in plasma resulted in coefficients of variation between 4 and 9% (intra-assay) and between 7 and 12% (inter-assay). Therefore, no loss of quality performance in the assay due to the modifications and the extensive sample preparation was observed. A method is considered precise for a single laboratory when quantitative analysis in concentrations below 1 mg/kg (1 ppm) results in a CV $<11\%$ (Prichard, Crosby, Day, Hardcastle, Holcombe, & Treble, 1995). The precision of the modified RPBA including sample pre-treatment therefore meets these requirements when used in foods with mainly 5-CH_3-H_4folate.

Table 5
Precision of the modified RPBA in selected food samples: intra- and inter-assay[a,b]

	Strawberries	CRM 485[d]	wpc 65
Intra assay[c]			
μg/100g	48.2	181.3	362
n	5	3	3
SD	2.1	2.3	23
CV%	4.4	1.2	6
Inter assay[d]			
μg/100g	46.5	186.1	380
n	7	3	3
SD	4.1	13.5	18
CV%	8.7	7.3	5

[a] Key: n = number of replicates, SD = standard deviation, CV% = coefficient of variation.
[b] CP conjugase used for strawberries and wpc 65, HK conjugase for CRM 485.
[c] Intra-assay: samples (3–5) prepared on the same day and analysed in the same RPBA assay.
[d] Inter-assay: duplicate samples prepared on individual days (3–7) and analysed in individual RPBA assays (3–7).

3.4. External method evaluation

3.4.1. Method comparison

Folate concentrations in selected foods were analysed using different quantification methods, for example, optimised RPBA, MA and HPLC (Table 6). Also, a comparison with previously reported HPLC folate data is presented. Results were similar for all tested food matrixes taking different sample preparation into account. The HPLC results confirm earlier studies showing that berries and milk mainly contain 5-CH_3-H_4folate (Vahteristo, Lehikoinen, Ollilainen, & Varo, 1997; Vahteristo, Ollilainen, & Varo et al., 1997; Wigertz & Jägerstad, 1995). According to authors named earlier, the folate content in strawberries and rose hips consists of 95% 5-CH_3-H_4folate or more and in milk at least 93% of the folate is 5-CH_3-H_4folate. The low HPLC result for milk in this study (3.4 μg/100 g, 90% 5-CH_3-H_4folate) may be because the HPLC method used is not optimised for milk samples and as

Table 6
Comparison of folate content (μg/100 g) in selected foods analysed using RPBA, microbiological assay and HPLC[a]

Method	Folate concentration μg/100 g		
	Strawberries	Rosehip (lyophilised)	Milk (skim)
RPBA			
Total folate	42±2 63±5[b]	259±7	5±03
MA			
Total folate	51	(274–408)	5
HPLC			
5-CH_3	47	251	3.1
H_4	n.d.	11	0.4
5-HCO	n.d.	n.d.	n.d.
PteGtu	n.d.	n.d.	n.d.
Total folate	45	253	3.4
Reported values	40±5[c] 65±14[d] 65±16[e]	210[f]	5±1[d] 5±1[g] 4±1[h]

[a] Key: H_4 = H_4 folate, 5-CH_3 = 5-CH_3–H_4 folate, 5-HCO = 5-HCO–H_4 folate, Total folate = PteGlu equivalents, n.a. = not analysed, n.d. = not detected RPBA: samples extracted as triplicates with CP conjugase and quantified in the same RPBA assay MA: Two of the three sample extracts quantified by RPBA HPLC: Samples extracted as duplicates with HK conjugase.
[b] Strawberry cultivar *Camorosa*.
[c] Vahteristo, Lehikoinen, Ollilainen, Koivistoinen, and Varo (1998).
[d] Konings, Roomans, Dorant, Glodbohm, Saris, and van den Brant (2001).
[e] Müller (1993).
[f] Levnedsmiddelstyrelsen (1996).
[g] Vahteristo, Ollilainen et al. (1997).
[h] Wigertz and Jägerstad (1995).

discussed earlier (Section 3.2), deconjugation of milk samples with HK-conjugase only may result in incomplete extraction of folate.

Total folate concentrations in rose hips quantified by MA, varied in this study from those from both RPBA and HPLC, as well as from one earlier reported MA value (Levnedsmiddelstyrelsen, 1996). Further investigation of the folate content in rose hips would be interesting, as we have shown that it is a rich source of folate. Of interest is also the significant difference in folate of the two strawberry cultivars (fresh *unknown* and frozen *Camorosa*) analysed in this study ($P < 0.01$), indicating either influence on folate concentrations by different varieties, cultivation, harvest and climate factors or by losses from storage and distribution. Variations from cultivars and sample handling could also explain contradictory results in literature (Konings, Roomans, Dorant, Goldbohn, Saris, & van den Brant, 2001; Müller, 1993; Vahteristo, Lehikoinen et al., 1997).

It is important to be careful when drawing conclusions from comparisons using different methods of quantification and sample pre-treatments. In the present study similar folate concentrations were obtained for selected foods using RPBA, MA and HPLC (Table 6). Future studies carrying out a method comparison with statistical analysis of results using the modified RPBA and other folate quantification methods commonly used to day would be interesting. No statistical evaluation was performed in this study due to the limited number of replicates, but the results indicated the modified RPBA to be suitable for folate analysis in tested foods. Also, the HPLC analyses showing berries and milk to contain mainly 5-CH_3-H_4folate (>90%) confirm the modified RPBA for folate quantification in this kind of products.

3.4.2. CRM materials

The amount of folates in CRM 421 was quantified after CP conjugase treatment with the modified RPBA and found to be 91 ± 5 μg/100 g ($n = 6$). The folate concentration obtained in CRM 485 was 204 ± 15 μg/100 g ($n = 9$), quantified after CP or HK conjugase treatment. The results for both CRM 421 and CRM 485 are lower than the reported values based on MA quantification: 142 ± 14 and 315 ± 28 μg/100 g, respectively (Finglas, Scott, Witthöft, van den Berg & de Froidmont-Görtz, 1999). This may be explained by the variable response to different folate forms using both quantification methods. CRM 421 is fortified with approx. 53% PteGlu (Finglas, Scott et al., 1999) and as described earlier this folate form has a 15% lower response than 5-CH_3-H_4folate in the modified RPBA. It has also been shown that 5-CHO-H_4folate does not respond at all in the RPBA. Indicative values for the 5-CH_3-H_4folate content in CRM 421 and CRM 485 are 25 and 214 μg/100 g, respectively (Finglas, Scott et al., 1999). Regarding quantification by MA, it is still not known whether test organisms respond equally to all native folates in biological material (Tamura, 1990). However, the total folate concentrations obtained using our RPBA are in the range of results from previously reported HPLC methods for both CRM 421 (92–106 μg/100 g) (Konings, 1999; Ndaw et al., 2001) and CRM 485 (186–214 μg/100g) (Konings, 1999; Finglas, Wigertz, et al., 1999).

4. Conclusions

A commercial RPBA was modified and quality controlled for analysis of food folate. Evaluated on berries and milk products, this RPBA with 5-CH_3-H_4folate calibration was found to be reliable for folate quantification in foods containing mainly 5-CH_3-H_4folate. Performance parameters, for example control of selectivity, absence of matrix effects, recovery (94–113%), precision (5–9 CV%, inter-assay) were satisfactory. However, the necessity of using external calibrated standards diluted in the same buffer as food samples to avoid unspecific binding to the folate binder when applying commercial RPBA kits for analysis of food folates has to be stressed. Thus, this modified RPBA has the potential to be a quick and inexpensive complement to HPLC and MA for folate quantification in foods containing mainly 5-CH_3-H_4folate.

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