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Biosensor-based determination of folic acid in fortified food

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Abstract

A newly developed method for the quantification of folic acid in fortified food is presented. An immunoaffinity-based optical biosensor was used to determine folic acid concentration levels in milk powder, infant formula and cereal samples. Accuracy of the method (88–101%) was demonstrated with the analysis of five reference samples. A collaborative precision study, where ten participants at four different laboratories analysed a set of ten samples, resulted in repeatability relative standard deviations of 2–8% and reproducibility relative standard deviations of 4–10%. © 2000 Elsevier Science Ltd. All rights reserved.

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1. Introduction

1.1. Fortification of food with folic acid

The addition of folic acid to certain cereal products like bread, flour, pasta and rice is required in several countries. The main argument for obligatory addition of folic acid to food is the possible prevention of prenatal neural tube defects resulting in spina bifida or anencephaly in new-born babies (Wald, 1991). Examples of other food products fortified with folic acid are infant formulae, breakfast cereals and fruit drinks. Pteroylglutamic acid (PGA) is used in fortification due to its stability (Ranhotra & Keagy, 1995), but it has been argued that 5-methyltetrahydrofolate (5-MTHF) may be a better alternative (BASF, 1998).

1.2. Analysis of folic acid in food

Added folic acid is determined in the quality and process control steps during food manufacturing, and as a part of legislative control of fortification at national authorities. Although PGA is used in fortification, natural folates may also be present in the product to be fortified (Rader, Weaver & Angyal, 1998). The simultaneous presence of several folate forms at low concentration levels puts great demands on the method of analysis. Further complications arise from the instability of several folates during sample preparation and the complexity of the sample matrices to be analysed. Problems associated with food folate analysis have been reviewed previously (Hawkes & Villota, 1989; Martin, 1995; Pfeiffer, Diehl & Schwack, 1994).

Microbiological assays are often regarded as reference methods for the analysis of some water-soluble vitamins in food. Turbidimetric growth measurements of *Lactobacillus casei* subsp. *rhamnosus* (ATCC 7469) are routinely used for the determination of total folate activity in deconjugase-treated sample extracts, but long analysis times and relatively low precision limit the usability of this type of assay (Ball, 1994; Wilson, Clifford & Clifford, 1987). Attempts to improve the performance of microbiological assays have been reported and include the use of microtitre plates for incubation (Horne & Patterson, 1988; Newman & Tsai, 1986), infrared (Goli & Vanderslice, 1989) or radioactivity measurement (Chen, Hill & McIntyre, 1983) of produced carbon dioxide, and the use of LASER in combination with differential light-scattering (Anderson, Angyal, Weaver, Felkner, Wolf & Worthy, 1993) to follow growth of the micro-organism. Alternatives to microbiological methods for folate analysis in food have been used for some time and include techniques based on liquid chromatography (Finglas, Wigertz, Vahteristo, Witthöft, Southon & de Froidmont-Görtz,

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1999; Lucock, Green, Priestnall, Daskalakis, Levene & Hartley, 1995; Pfeiffer, Rogers & Gregory, 1997; Vahteristo, Ollilainen, Koivistoinen & Varo, 1996), and the use of folate-binding proteins (Finglas, Kwiatkowska, Faulks & Morgan, 1988) and anti-folate antibodies (Reichert & Rubach, 1991). A number of assay kits are commercially available for folate analysis. They are almost exclusively intended for the analysis of clinical samples and differences in sample composition and selectivity for folate forms make few of them suitable for the analysis of folates in food (Finglas, Faure & Southgate, 1993). The comparison of food folate methods with different principles of analysis has been reported (Finglas et al., 1988; 1993; Österdahl & Johansson, 1989; Seale & Finglas, 1995; Wigertz & Jägerstad, 1995).

Despite the efforts devoted to develop folate methods with better performance, there are still problems to be solved. Many methods are time-consuming and the precision achieved may be strongly dependent on the operation of skilled personnel. Unwanted interference from the sample matrix often necessitates the use of extensive sample extraction and clean-up (Finglas, van den Berg & de Froidmont-Görtz, 1996; Pfeiffer et al., 1997; Seale & Finglas, 1995; Wigertz, Svensson & Jägerstad, 1997). A more rapid and simple method for determination of folic acid in fortified food would be advantageous in terms of reduced time from sample to results, as well as less demands on skilled personnel. The need for this will be even more important as more and more food products are fortified with folic acid (DeVries & Nelson, 1994). A fast and easy extraction procedure would also be preferred, since folates are sensitive to light exposure, oxidation and extreme pH (Pfeiffer et al., 1994; Vahteristo et al., 1996; Wigertz et al., 1997).

1.3. Biosensor-based determination of folic acid

A new technique for quantification of folic acid in fortified food has been developed: an immunoassay performed in an optical biosensor system which utilises the phenomenon of surface plasmon resonance (SPR) as detection principle (Jönsson et al., 1991; Löfås & Johnsson, 1990; Stenberg, Persson, Roos & Urbaniczky, 1991). Several factors contribute to making the method rapid and easy to use e.g. the sensitive SPR detection, the specific interaction between analyte and antibody and the low non-specific binding. Measurements may be performed in coloured, turbid or opaque solutions, and there is no need of any colour reagents or extended incubation times. The biosensor is a continuous-flow system, with an automatic sample handling. A run is completed within 12 h including sample preparation and measurement of calibrants and 40 samples. The results are presented continuously.

The biosensor system has earlier shown good performance in various application areas for quantitative as well as qualitative determinations in food, e.g. antibiotics (Baxter, O'Connor, Haughey, Crooks & Elliot, 1997; Bergström, 1998; Crooks, Baxter, O'Connor & Elliot, 1998; Mellgren & Sternesjö, 1998; Mellgren, Sternesjö, Hammer, Suhren, Björck & Heeschen, 1996; Sternesjö, Mellgren & Björck, 1995), mycotoxins (van der Gaag, Stigter, van Duijn, Bleeker, Hofstra & Wahlström, 1997), pesticides (Minunni & Mascini, 1993; Wagner, Bindler & Gadani, 1995), food-borne pathogens (Fratamico, Strobaugh, Medina & Gehring, 1998; Haines & Patel, 1995) and β-agonist residues (Elliot et al., 1998; Hellenäs, Johansson, Elliot & Hewitt, 1997).

The objective of this study was to validate this new biosensor-based method for the quantification of folic acid in fortified food, in terms of accuracy and precision.

2. Materials and methods

2.1. Instrumentation

The analytical data reported in this study were produced with BiacoreQuant™ biosensor systems (Biacore AB, Uppsala, Sweden). In the precision study six preproduction units situated at four different laboratories were used (one laboratory in Sweden [three units], one laboratory in the Netherlands [one unit], and two laboratories in Switzerland [one unit each]). One production unit situated at one laboratory (in Sweden) was used in the accuracy study.

2.2. Folic acid kit

Ready-to-use analysis kits for the quantification of folic acid were supplied from Biacore AB (Uppsala, Sweden). In the precision study pre-production kits were used, while a production kit was used in the accuracy study. Each kit included folic acid stock solution (PGA), anti-folic acid antibody, folic acid sensor chip, regeneration solution, conditioning solution and disposable materials (e.g. microtitre plate, cover for microtitre plate, vials and septa). All analyses were performed within the four weeks of satisfactory kit performance guaranteed by the manufacturer.

2.3. Samples

The accuracy study was performed using the cereal samples VMP 1/1998, VMP 2/1998, VMP 3/1998 and VMP 4/1998 from the AACC® Check Sample Program (American Association of Cereal Chemists, St Paul, MN, USA) and the milk-based infant formula NIST SRM® 1846 (National Institute of Standards & Technology, Gaithersburg, MD, USA). A number of sample types were used for the precision study, representing milk powder, cereal, milk-based infant formula, soy-based infant

formula and vitamin premix. Ten samples in duplicate were supplied pre-weighed in glass-vials to each participant.

2.4. Extraction procedure

The sample (0.3–1 g) was dissolved in 40–1000 ml water (resistivity $\geqslant$18 MΩ/cm, e.g. using 0.22 μm Milli-Q®, Millipore Ireland BV, Cork, Ireland), producing an approximate folate concentration of 7–80 ng/ml in the extracts. All samples except the milk-based infant formula in the accuracy study were ultra-sonicated for 15 min (e.g. using Branson 5210, Branson Ultrasonics BV, Soest, The Netherlands). The milk-based infant formula in the accuracy study was autoclaved at a temperature of +105°C for 10 min (Certoclav Multicontrol, Certoclav Sterilizer GesmbH, Traun, Austria). All extracts were centrifuged in 2-ml Eppendorf vials for 10 min (e.g. using Hettich Mikroliter centrifuge, Hettich Zentrifugen, Tuttlingen, Germany at 15 000 rpm), and finally the supernatants were filtered through 0.22-μm syringe filters (MILLEX®)-GS, Millipore S.A., Molsheim, France).

2.5. Analysis

2.5.1. Detection principle

A biosensor may be defined as an instrument that combines a biological recognition mechanism with a sensing device or transducer. The optical biosensor used in this study is a fully automated continuous-flow system, which exploits the phenomenon of surface plasmon resonance (SPR) to detect and measure biomolecular interactions (Fig. 1). The essential components of the biosensor system are: the sensor chip with a biospecific surface, the liquid handling flow system with an autosampler, precision pumps and an integrated μ-fluidic cartridge (IFC), and the optical detection unit.

The sensor chip is the signal transducer in the biosensor system, i.e. this is where the biomolecular interactions take place. The sensor chip consists of three layers: glass, a thin gold film and a dextran matrix to which the analyte is immobilised. The dextran layer increases sensitivity by increasing the binding capacity of the surface and it provides a very low degree of non-specific binding to the surface.

The autosampler is responsible for the sample handling, including mixing and transferring of samples and reagents to mixing-positions in the microtitre plate or to the IFC injection port. A smooth pulse-free flow through the system is achieved with the help of two specially designed syringe pumps; one for buffer flow and one for the autosampler functions. The IFC contains flow channels, sample loops and pneumatic valves, and controls delivery of solutions to the sensor surface. By pressing the IFC against the sensor chip, the flow cells for detection are formed. The continuous-flow technology with microfluidics allows rapid switching between sample and buffer at the sensor surface, resulting in minimum usage of the sample.

The optical detection unit is responsible for generation and detection of the SPR signal. SPR occurs when surface plasmon waves are excited at the metal/liquid interface of the sensor chip under conditions of total internal reflection. A marked drop in intensity of the reflected light at a specific angle is detected. This resonance angle is very sensitive to the refractive index of

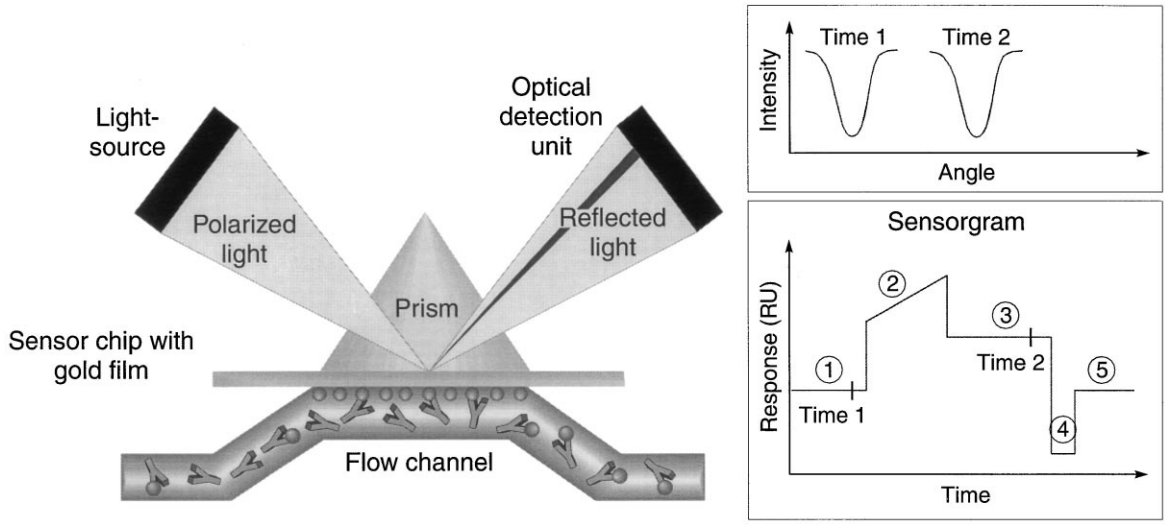

Fig. 1. Principle of surface plasmon resonance (SPR) detection. SPR occurs when surface plasmon waves are excited at the metal/liquid interface of the sensor chip under conditions of total internal reflection. A marked drop in intensity of the reflected light at a specific angle, the resonance angle, is detected. Changes in the refractive index of the solution close to the sensor chip surface, e.g. after biomolecular interactions, will change the resonance angle and can be measured as a change in the SPR signal (expressed in resonance units, RU). The SPR signal is plotted against time in a sensorgram, and the different phases during a typical analytical cycle are illustrated: 1. baseline (time 1); 2. binding; 3. response plateau (time 2); 4. regeneration; 5. back to baseline.

the solution close to the sensor chip surface. Changes in the refractive index, e.g. after biomolecular interactions, will change the resonance angle and can be measured as a change in the SPR signal (expressed in resonance units, RU). Comprehensive descriptions of the technique and the detection principle are given elsewhere (Biacore, 1998b; McCormack, Keating, Killard, Manning & O'Kennedy, 1998).

2.5.2. Assay principle

The folic acid assay is designed as an inhibition assay (Fig. 2). An excess of specific antibodies is mixed by an automated procedure with sample or calibration solution containing the free analyte, and the antibody and analyte form a complex. When injected into the detection unit, non-complexed antibodies are measured by the biosensor system when they bind to the analyte immobilised on the sensor chip. At the end of each analytical cycle the sensor surface is prepared for a new sample by injection of a regeneration solution that dissociates the analyte/antibody complex on the surface.

2.5.3. Specificity

The specificity of the anti-folic acid antibody, as given by the kit manufacturer, is shown in Table 1.

2.6. Validation

2.6.1. Accuracy

Folic acid was measured in four cereal samples and a milk-based infant formula. Single determinations of five separate preparations for each sample were performed in one run. Average, standard deviation, relative standard deviation, 95% confidence interval and accuracy in relation to expected values were calculated for all samples.

2.6.2. Precision

The precision of the method was evaluated in a collaborative study with ten participants at four different laboratories, following the procedure recommended in the IUPAC/ISO/AOAC INTERNATIONAL Harmonisation Protocol on Collaborative Studies (Horwitz, 1995). More detailed information on the participants is presented in Table 3. Samples were analysed as blind replicates or at double-split level, i.e. as blind replicates of two different samples with similar composition and approximately the same content of folic acid.

Table 1
Relative specificity of the anti-folic acid antibody used in the biosensor kit (Biacore, 1998a)

Substance	Cross reactivity (%)
PGA	100
5-Methyltetrahydrofolate	100[a]
Dihydrofolate	17
Tetrahydrofolate	8
5-Formyltetrahydrofolate	0

[a] In the presence of 1% sodium ascorbate during extraction.

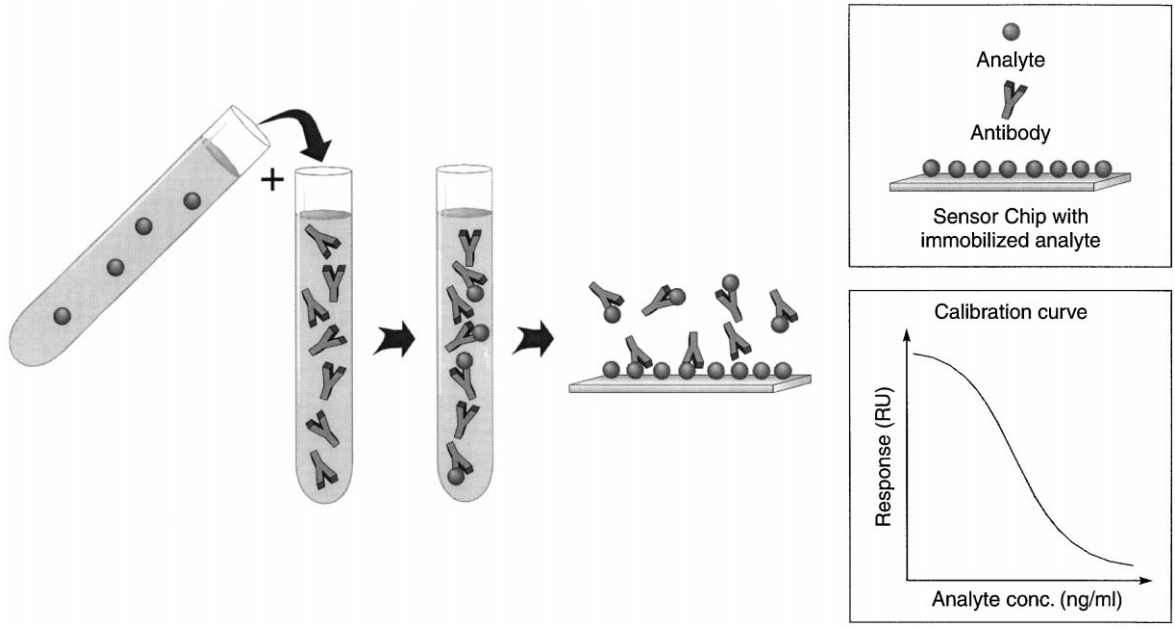

Fig. 2. Principle of inhibition assay. An excess of specific antibodies is mixed by an automated procedure with sample or calibration solution containing the free analyte, and the antibody and analyte form a complex. When injected into the detection unit, non-complexed antibodies are measured by the biosensor system when they bind to the analyte immobilised on the sensor chip. The biosensor response will be inversely proportional to the analyte concentration.

3. Results and discussion

3.1. Accuracy

Accuracy for the five samples studied are given in Table 2 and Fig. 3 and ranged from 88 to 101%, relative to the reference values. The accuracy study shows that the folic acid content in the samples measured with the biosensor-based assay is well in accordance with that obtained with the reference techniques. Further studies demonstrating good agreement between the biosensor-based folic acid assay and reference microbiological methods have been reported elsewhere (Indyk et al., 2000; Lindeberg & von Malmborg, 1998).

The different sample preparation for the cereals versus the milk-based infant formula was due to the residual amounts of folate-binding proteins (FBP) in milk samples. A fraction of FBP in the sample is often free and

Table 2
Demonstration of accuracy of the biosensor method using five reference samples

	VMP 1 (μg/100 g)	VMP 2 (μg/100 g)	VMP 3 (μg/100 g)	VMP 4 (μg/100 g)	NIST (μg/100 g)
Measured folic acid	377	501	746	1280	123
	385	498	744	1290	124
	391	498	742	1310	123
	399	511	751	1310	124
	402	501	740	1270	122
Average	390.8	501.8	744.6	1292	123.2
SD	10.2	5.4	4.2	17.9	0.8
RSD	2.6%	1.1%	0.6%	1.4%	0.7%
Found (95% CI)	391±13[a]	502±7[a]	745±5[a]	1292±22[a]	123±1[a]
Expected (95% CI)	443±29[b]	497±57[b]	810±55[b]	1370±160[b]	129±28[c]
Accuracy[d]	88%	101%	92%	94%	96%

[a] Five determinations on separate sample preparations in one run.

[b] Ten to twelve separate determinations at different laboratories by different analysis techniques. The expected values with 95% CI were calculated from the values given in the respective AACC® Check Sample report.

[c] Six separate determinations at different laboratories by microbiological assay. The expected value with 95% CI was taken from the certificate for NIST SRM® 1846.

[d] Accuracy was calculated as the mean folate content found in this study divided by the relevant expected value (see notes b and c).

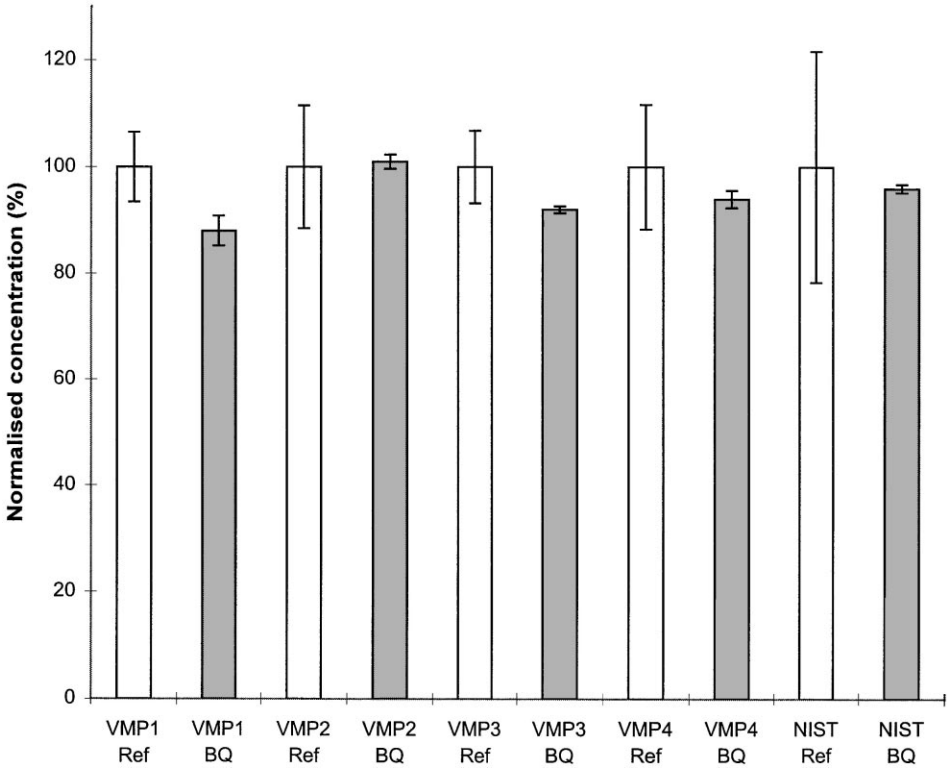

Fig. 3. Comparison between folic acid results with biosensor system (BQ) and with other techniques (Ref), where all expected values are set to 100%.

able to bind to added folic acid. The amount of active FBP capable of binding folic acid is dependent upon heat treatment during manufacture of the product (Wigertz, 1997; Wigertz et al., 1997; Wigertz, Hansen, Høier-Madsen, Holm & Jägerstad, 1996). To enable analysis in these matrices the folic acid has to be liberated from the FBP, for example by protein denaturation. Recent investigations show that heat treatment at 105°C for 10 min, or in a boiling water bath for 15 min efficiently denatures the proteins (Boström, Wahlström & Persson, 1998).

3.2. Precision

The participants' results from the analysis of the samples in the collaborative precision study are reported in Table 3. The statistical evaluation of the collaborative precision study was performed according to the IUPAC/ISO/AOAC INTERNATIONAL Harmonisation Protocol on Collaborative Studies (Horwitz, 1995) and included Cochran, single Grubbs and paired Grubbs tests for outliers. One Cochran outlier ($P = 0.025$) was identified and the result of the statistical evaluation is presented in Table 4.

The repeatability relative standard deviation (RSD_r) for the biosensor-based determination of folic acid in fortified food ranged between 2 and 8% and the reproducibility relative standard deviation (RSD_R) ranged between 4 and 10%. The RSD_R values of 29% for Premix 2 and 12% for Milk powder 2 can probably be explained by inhomogeneity of these samples. The precision values achieved compare favourably with a collaborative study including 13 laboratories determining folic acid with microbiological assay (Association of Official Analytical Chemists [AOAC], 1995) in three samples of milk-based infant formula, reporting a RSD_r value of 9.35% and a RSD_R value of 25.44% (Tanner, Barnett & Mountford, 1993). When the RSD_R values from the present study were divided by the corresponding predicted RSD_R values ($PRSD_R$) calculated according to Horwitz (Albert & Horwitz, 1997; Horwitz, 1982; Horwitz, Kamps & Boyer, 1980), most of the resulting HORRAT values were in the lower end of the acceptable region of 1.0±0.5 (Horwitz, Britton & Chirtel, 1998). The very low ratios (0.3–0.4) for Milk powder 1, Milk-based infant formula 2 and Soy-based infant formula 1 may partly be explained by the fact that the ten participants used only six different instruments at four different laboratories, but can also be seen as a confirmation of the inherently high precision of the analysis technique. Premix 2 had a high HORRAT value of 3.8, probably because of inhomogeneity as discussed below.

Since not all participants in the study were truly independent, the results were recalculated taking into

Table 3
Folic acid concentrations in ten samples for the collaborative precision study as measured using the biosensor system (μg/100 g)

	Information on participants									
Participant	A	B	C[a]	D[a]	E	F	G	H	I	J
Laboratory	1	2	1	1	3	1	1	1	1	4
Instrument	a	b	c	a	d	a	e	e	a	f
Instrument experience	No	Yes	Yes	Yes	Yes	No	No	No	No	Yes
Vitamin experience	No	Yes	No	No	Yes	Yes	No	Yes	No	Yes
Cereal 1	323	322	298	306	314	303	287	337	316	342
	323	305	305	307	290	324	256	331	297	328
Cereal 2[b]	305	284	289	292	298	298	275	310	285	335
	181	286	291	289	280	285	258	310	277	334
Milk powder 1	57.3	54.3	51.6	54.6	53.6	60.4	54.0	59.2	55.6	61.2
	51.6	58.7	57.4	54.4	54.5	57.9	56.0	59.2	56.0	58.9
Milk powder 2	35.4	37.6	29.6	29.9	28.8	36.3	24.4	34.7	29.7	34.1
	38.6	33.3	31.5	31.8	27.8	38.5	26.6	34.8	33.1	32
Premix 1	13900	11400	13100	12100	14100	13700	13400	14500	13000	15300
	13400	10900	14500	12700	13900	13700	13200	14000	13300	15000
Premix 2	16000	14300	14400	6950	5940	14200	10800	16300	16300	15400
	16300	12600	12400	8080	5250	16100	12500	14900	16800	14900
Milk-based infant formula 1	50.0	52.9	45.6	40.4	45.3	50.5	40.2	42.6	40.3	51.3
	50.6	49.7	44.2	44.1	45.6	53.7	41.2	43.4	40.2	46.8
Milk-based infant formula 2	58.2	61.7	58.1	49.7	46.5	60.8	56.3	58.8	57.5	60.8
	57.6	54.6	58.7	52.0	48.1	58.2	54.2	57.8	55.0	62.4
Soy-based infant formula 1	66.2	63.9	61.4	59.5	63.0	68.0	61.1	66.6	63.1	66.3
	64.7	66.2	61.9	59.7	67.1	68.2	62.2	65.6	59.6	65.4
Soy-based infant formula 2	41.4	51.9	38.3	40.2	42.9	42.5	38.2	41.2	36.8	43.2
	40.3	40.8	38.7	41.2	44.9	40.4	32.4	40.6	36.7	38.1

[a] Participants C and D performed the analyses on the same day.
[b] Participant A is a Cochran outlier ($P = 0.025$) for Cereal 2.

Table 4
Statistical evaluation of the collaborative precision study — all participants

Material	Data	No. of labs	No. of tests	Statistics								
				Mean (μg/100 g)	s_r (μg/100 g)	s_R (μg/100 g)	RSD_r (%)	RSD_R (%)	r	R	$PRSD_R$[a] (%)	HORRAT
Cereal 1	All	10	20	311	12	20	3.9	6.5	34	57	13	0.48
Cereal 2	Outlier removed (A)	9	18	293	7	20	2.4	6.8	19	56	14	0.50
Milk powder 1	All	10	20	56.3	2.3	2.8	4.0	5.0	6.3	7.8	17	0.29
Milk powder 2	All	10	20	32.4	1.8	4.0	5.5	12	5.0	11	19	0.63
Premix 1	All	10	20	13500	400	1100	3.0	8.3	1100	3100	8	1.09
Premix 2	All	10	20	13000	900	3800	7.2	29	2600	10600	8	3.77
Milk-based infant formula 1	All	10	20	45.9	1.7	4.6	3.7	10	4.8	12.9	18	0.56
Milk-based infant formula 2	All	10	20	62.6	2.1	4.5	3.4	7.2	5.9	12.7	17	0.42
Soy-based infant formula 1	All	10	20	64.0	1.4	2.9	2.2	4.5	4.0	8.1	17	0.26
Soy-based infant formula 2	All	10	20	40.5	3.1	3.9	7.7	9.6	8.7	10.9	18	0.52

[a] $PRSD_R\ (\%) = 2^{(1-0.5\log C)}$, where C is the relative analyte concentration in mass/mass units; $HORRAT = RSD_R/PRSD_R$.

account laboratory and instrument identity. The results are presented in Tables 5 and 6. Although RSD_r- and RSD_R-values were generally higher for truly independent participants ($n=4$), the quotient r/R showed no clear difference as compared to where participants used the same instrument in the same laboratory ($n=4$). HORRAT values were also higher but still stayed low at around 0.5, Premixes 1 and 2 excluded. It can be concluded that the method performance indicated in the results of the precision study was not influenced to a great extent by the fact that participants were not truly independent.

The precision study reported herein was initially designed for samples at double-split level. Samples with close resemblance, but at a slightly different concentration of folic acid, were prepared by the organisers simply by dry mixing of two samples of different folic acid levels. The results show that samples prepared in this way were also the ones with the highest deviation (Premix 2 and Milk powder 2), indicating that sample homogeneity was insufficient. Since the samples also were not identical in composition, and the achieved folic acid concentration in two out of three cases were more than three standard deviations from that of the original sample, it was decided to calculate the precision as if the samples were not double-split. Instead all samples were evaluated as blind replicates. Another possible source of error was the use of pre-mixes that had been exposed to air, moisture, and light several times. In addition, it was observed by many of the operators that the pre-mix samples did not dissolve properly.

The kit manufacturer's recommendations for sample preparation prior to analysis have been improved since the precision study was performed with pre-production kit and instrumentation (Biacore, 1998a). Present recommendations are e.g. to dissolve the sample in a

Table 5
Statistical evaluation of the collaborative precision study — same laboratory, same instrument

Material	Data	No. of labs	No. of tests	Statistics								
				Mean (μg/100 g)	s_r (μg/100 g)	s_R (μg/100 g)	RSD_r (%)	RSD_R (%)	r	R	$PRSD_R$[a] (%)	HORRAT
Cereal 1	A, D, F, I	4	8	312	10	10	3.2	3.4	28	30	13	0.25
Cereal 2	D, F, I	3	6	288	6	7	2.2	2.5	18	20	14	0.19
Milk powder 1	A, D, F, I	4	8	56.0	2.2	2.7	3.9	4.8	6.2	7.6	17	0.28
Milk powder 2	A, D, F, I	4	8	34.2	1.9	3.8	5.7	11	5.4	10.6	19	0.59
Premix 1	A, D, F, I	4	8	13200	300	600	2.2	4.8	800	1800	8	0.63
Premix 2	A, D, F, I	4	8	13800	800	4300	5.8	31	2300	12000	8	4.07
Milk-based infant formula 1	A, D, F, I	4	8	46.2	1.7	6.0	3.8	13	4.9	16.7	18	0.72
Milk-based infant formula 2	A, D, F, I	4	8	56.1	1.5	3.9	2.7	7.0	4.3	11.0	17	0.40
Soy-based infant formula 1	A, D, F, I	4	8	63.6	1.4	4.0	2.1	6.3	3.8	11.1	17	0.37
Soy-based infant formula 2	A, D, F, I	4	8	39.9	0.9	2.2	2.3	5.6	2.5	6.3	18	0.31

[a] $PRSD_R\ (\%) = 2^{(1-0.5\log C)}$, where C is the relative analyte concentration in mass/mass units; $HORRAT = RSD_R/PRSD_R$.

Table 6
Statistical evaluation of the collaborative precision study — different laboratories, different instruments

Material	Data	No. of labs	No. of tests	Statistics								
				Mean (μg/100 g)	s_r (μg/100 g)	s_R (μg/100 g)	RSD_r (%)	RSD_R (%)	r	R	$PRSD_R$[a] (%)	HORRAT
Cereal 1	C, B, E, J	4	8	313	12	18	3.8	5.7	33	50	13	0.42
Cereal 2	C, B, E, J	4	8	300	6	24	2.2	7.9	18	67	14	0.59
Milk powder 1	C, B, E, J	4	8	56.3	2.7	3.3	4.8	5.9	7.6	9.4	17	0.34
Milk powder 2	C, B, E, J	4	8	31.8	1.9	3.4	5.8	11	5.2	9.4	19	0.56
Premix 1	C, B, E, J	4	8	13500	500	1700	4.0	13	1500	4900	8	1.68
Premix 2	C, B, E, J	4	8	11900	1000	4300	8.2	36	2700	12100	8	4.68
Milk-based infant formula 1	C, B, E, J	4	8	47.7	2.0	3.4	4.2	7.0	5.6	9.4	18	0.39
Milk-based infant formula 2	C, B, E, J	4	8	56.4	2.6	6.5	4.7	12	7.4	18.2	17	0.66
Soy-based infant formula 1	C, B, E, J	4	8	64.4	1.7	2.2	2.6	3.5	4.8	6.2	17	0.20
Soy-based infant formula 2	C, B, E, J	4	8	42.4	4.4	4.7	10	11	12.3	13.0	18	0.60

[a] $PRSD_R\ (\%) = 2^{(1-0.5\log C)}$, where C is the relative analyte concentration in mass/mass units; $HORRAT = RSD_R/PRSD_R$.

volumetric flask to get an exact extract volume instead of adding a certain volume to the sample, to prepare the calibration solutions from a stock solution the same day as the analysis instead of using ready-made calibration solutions, to use no less than one gram of sample, to autoclave milk samples at +105°C for 10 min, and to extract the sample in 1% sodium ascorbate if a high concentration of 5-MTHF is expected.

4. Conclusions

The validation of a new biosensor-based method for the quantification of folic acid in fortified food is presented. Accurate results were achieved as determined by the analysis of reference samples (NIST and AACC). Precision values within and between laboratories compare favourably to those achieved with other methods of analysis. The sample preparation is simple and rapid, with low demands on skilled personnel and hands-on time. A run is completed within 12 h including sample preparation and measurement of 40 sample extracts. These factors all contribute to making the biosensor system an attractive alternative to more tedious and less precise assays.

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