Food Chemistry 49 (1994) 191–201
Analytical Methods Section

Application of biospecific methods to the determination of B-group vitamins in food—a review

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(Received 20 February 1993; revised version received and accepted 14 April 1993)

Biospecific methods using either antibodies or naturally occurring vitamin binding proteins for the determination of pantothenic acid, biotin, folate and vitamins B_6 and B_{12} in food are reviewed. Both types of assay are based on the 96-well microtitration plate format, which offers the potential for high sample throughput. Another feature of these techniques is their flexibility to produce either highly specific assays (suitable for the analysis of individual forms), or much broader specificity assays where the analysis of groups of vitamins is required. The availability of both polyclonal and monoclonal vitamin antisera is also outlined. The ELISA format using antibodies has a greater potential for the detection of specific vitamers whereas EPBAs are useful for assays of broad specificity where the analysis of a group of vitamers is required, as in the case of folate or biotin.

INTRODUCTION

There is a growing need for more rapid and specific methods for vitamin analysis, in particular to meet the demands of nutritional labelling of foods and other nutritional studies. As an alternative to the more conventional methods of analysis biospecific procedures using either antibodies or vitamin binding proteins are well-suited to meeting these demands.

Biospecific methods of analysis are used to describe two distinct types of procedure: firstly, those based on the specific interaction of an antibody with its antigen, for example, the radioimmunoassay (RIA) or the enzyme-linked immunosorbent assay (ELISA); secondly, those based on the use of naturally occurring vitamin binding proteins with either radiolabels (as in the radiolabelled protein binding assay, RPBA) or enzyme labels (as in the enzyme protein binding assay, EPBA). The RPBA is a variant of the isotope-dilution method and is often referred to as a 'radioassay'. There are several commercially available 'radioassay' kits for the determination of vitamins in blood samples, but their application to food analysis is limited. Examples of biospecific methods used for the determination of vitamins are given in Table 1.

Although RIA has been useful technique in clinical analysis, it has the major disadvantage that it uses radioactivity, which presents problems associated with its safe-handling and disposal. This makes the use of RIA in routine food analysis unattractive. In addition, scintillation counting of the radioactivity can be slow and expensive.

The ELISA performed in the plastic wells of microtitration plates has the advantage of utilising stable and non-reactive reagents which can be stored for long periods. These assays can be readily amenable to automation but can also be carried out with inexpensive equipment. This type of immunoassay has been widely exploited in food analysis (Morgan, 1985). The type of ELISA system commonly used for vitamins is the indirect type, where vitamin–protein conjugate (known as the hapten) is immobilised to the well surface. The protein used is different from that used for the immunogen. The format for this type of assay is shown in Fig. 1.

Primary anti-vitamin antibody and vitamin are added to each well and the antibody becomes distributed between immobilised and free vitamin according to how much free vitamin is present initially. After phase separation, achieved by well emptying and washing, bound primary antibody is detected by the addition of a species-specific, enzyme-labelled second antibody. These are readily available commercially, active against different species and labelled with a variety of enzymes. After an appropriate incubation period, excess unbound material is removed and substrate added. Well optical densities are determined after a certain time and

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Table 1. Biospecific methods of vitamin analysis in food and biological materials

Type	Vitamin	Reference
Radioimmunoassay (RIA)	Pantothenic acid	Wyse *et al.* (1979)
	Pteroylmonoglutamic acid (PGA)	DaCosta & Rothenberg (1971); Hendel (1981)
	25-OH-D_3[a]	Incstar Ltd (Incstar Corporation, Stillwater, MI 55080, USA)
Enzyme-linked immunosorbent assay (ELISA)	Pantothenic acid	Morris *et al.* (1988)
	Biotin	Alcock
	Pyridoxamine (PAM)	Alcock *et al.* (1990)
	Folate	Finglas, P. M. & Morgan, M. R. A., unpublished
Radiolabelled protein binding assay (RPBA)	Vitamin B_{12}	Lau *et al.* (1965)
	5-methyl-THF	Gutcho & Mansbach (1977)
	Biotin	Hood (1975); Dakshinamurti & Allan (1979)
	25- & 24,25-OH-D_3	Reinhardt & Horst (1988)
	1,25-$(OH)_2$-D_3	Reinhardt *et al.* (1984)
Enzyme protein binding assay (EPBA)	Vitamin B_{12}	Alcock *et al.* (1992)
	Biotin	Finglas *et al.* (1986)
	Folates	Finglas *et al.* (1888*b*)

[a] Commercially available kit.

unknown samples quantified by reference to standard curves.

The antibody is the most important component of any immunoassay system (such as RIA and ELISA), and antibody specificity is usually dependent on the immunogen used. Vitamins alone as small molecules (low molecular weight) are not immunogenic, i.e. they do not stimulate antibody production in animals. In order to obtain antibodies, the vitamin must first be coupled to a larger, immunogenic carrier molecule, normally a protein. The way in which the vitamin–protein conjugate is produced, i.e. the use of different functional groups to couple to the protein, is crucially important in defining the specificity of the antisera produced.

The coupling of the vitamin to the protein usually involves linking a carboxylic acid group to an amino group on the protein either by the carbodiimide reaction (Goodfriend *et al.*, 1964), or by the mixed anhydride procedure (Erlanger *et al.*, 1959). Alternatively, if there is no carboxylic acid group available on the vitamin, then one can be produced by modification of a hydroxyl or a keto group. Other ways of coupling vitamins to proteins include the periodate method which can be used for carbohydrate residues (Butler & Chen, 1967) and bromoacetyl bromide procedure for primary alcohol groups (Wyse *et al.*, 1979).

The antibody generated will recognise most strongly the part of the hapten (coupled vitamin compound) which is distal to the site of the conjugation to the protein. Much less recognition will be shown to compounds that differ in structure only at or near to the point of conjugation. This is especially important for vitamins where a number of closely related compounds (or vitamers) may exist in the food, and the analysis of one or more of these forms is needed for the total vitamin activity of the food. Considerable attention, therefore, should be given to the choice of coupling procedure used for the synthesis of the hapten–protein conjugates so that antisera of the required specificity can be produced.

The production of suitable antisera can be a lengthy procedure and can take several months. However, the procedure is fairly simple to perform involving the immunisation of animals, usually rabbits, with the hapten–protein conjugate mixed in Freund's adjuvant, which is used to prolong and enhance the immune

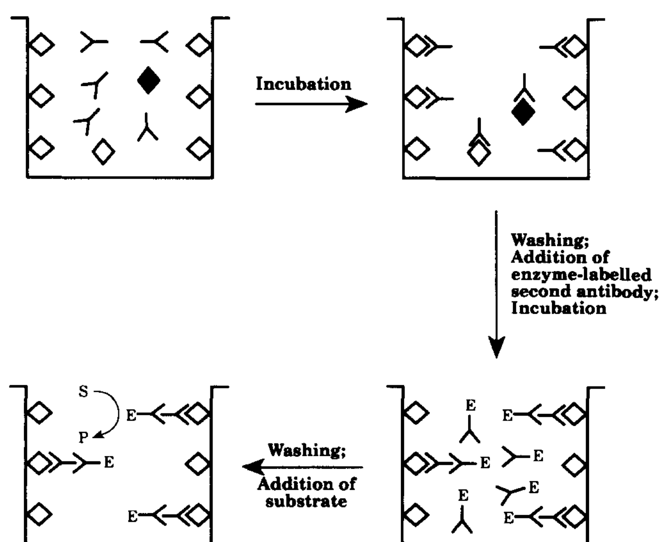

Fig. 1. Indirect ELISA performed on a microtitration plate. Y, primary antibody; ♦, immobilised vitamin; ◇, free vitamin; E, enzyme labelled second antibody; S, substrate; P, coloured end-product. [From: Kemp, H. A. & Morgan, M. R. A. (1987). Use of immunoassays in the detection of plant cell products. In: *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 4, ed. Academic Press, London, pp. 287–302.]

Table 2. Commercially available vitamin antisera

Antibody to	Type	Host animal	Specificity
D-Biotin	Polyclonal, enzyme labelled (alkaline phosphatase, peroxidase)	Goat	D-Biotin, avidin (?)
Vitamin B_{12}	Monoclonal (IgG1)	Mouse	Free and protein bound biotin, adenosylcobalamin
Folic acid	Monoclonal (IgG2b)	Mouse	Free and protein bound PGA + 5-methyl-THF
Folic acid	Polyclonal	Sheep	Free or protein bound PGA
5-Methyl-THF	Monoclonal (IgG2b)	Mouse	Free or protein bound 5-methyl-THF
5-Methyl-THF	Polyclonal	Sheep	Free or protein bound 5-methyl-THF
5-Formyl-THF	Polyclonal	Sheep	Free or protein bound 5-formyl-THF
Vitamin B_{12}	Polyclonal	Sheep	Vitamin B_{12}

response in the animals. Amounts of conjugate injected can vary from as little as 100 μg per animal to much larger amounts. After a booster injection, normally 4–6 weeks after the primary injection, the animal is bled and the serum checked for the required antibody content, avidity and specificity. The animals can be further boosted and bled until the required antiserum is obtained. In the case of antibody production to pantothenic acid, five to six booster injections at 6-weekly intervals were needed before a suitable antiserum was obtained (Morris *et al.*, 1988).

The antibodies produced by the above procedures are polyclonal, i.e. the antiserum consists of a large number of different antibodies against the hapten, each with slightly different properties relative to it.

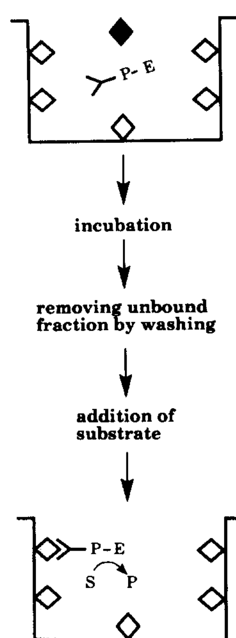

Fig. 2. EPBA format performed on a microtitration plate. P–E, enzyme–vitamin binding protein conjugate; ♦, free vitamin; ◇, immoblised vitamin; S, substrate; P, coloured end-product.

Monoclonal antibodies have also been developed (Kohler & Milstein, 1975) which are produced *in vitro* from one cell line or clone and are therefore identical. The advantages of using monoclonal antibodies are that they are available in unlimited amounts and that the method of production can sometimes result in an antibody of improved characteristics.

Most of the antibodies to vitamins are polyclonal, although there are commercially available monoclonal antibodies to specific vitamin forms (see below). Commercially available antisera (both polyclonal and monoclonal) to a range of vitamin forms have also been developed primarily for clinical use (Table 2). Although antisera specificities have been given in most cases, their application in the ELISA format to the analysis of vitamins in food has not been examined fully.

The use of naturally occurring vitamin binding proteins in place of the antibody can also result in suitable assays. These assays exhibit slightly different characteristics to the antibody based procedures. However, most of the advantages of ELISA are applicable to PBA. Both assay systems are based on the 96-well microtitration plate format, which is extremely easy to use and ideal for the batchwise analysis of food (Finglas & Morgan 1988*a*).

In the case of EPBA, the binding protein is labelled directly by chemically coupling to an enzyme such as horseradish peroxidase. The protein–enzyme replaces the antibody, and can be used either directly in the assay (after appropriate dilution), or after further purification. The format for the EPBA is similar to the ELISA but contains one incubation step less (Fig. 2).

The development and application of biospecific methods has largely focused on the water-soluble, B-group vitamins, where existing methodology is in most need of improvement. For these vitamins a range of binding proteins are readily available and a number of RPBAs have been used for clinical work. The assays for the individual vitamins will be discussed in more detail as follows.

APPLICATION OF BIOSPECIFIC METHODS

Pantothenic acid

Pantothenic acid occurs in food in the free form and in larger amounts bound predominantly as coenzyme A and other protein derivatives (Fox, 1984). Much of the data on the pantothenate content of food has been obtained previously by microbiological assay using *Lactobacillus plantarum* after a double enzyme extraction procedure to release the bound vitamin forms.

The development and validation of a radioimmunoassay for free pantothenic acid was first reported in the late 1970s (Wyse *et al.*, 1979). This procedure was based on the competitive binding of an antibody specific for pantothenic acid between the unlabelled vitamin in the sample or standard and radiolabelled antigen. Although the assay was found to be specific and sensitive for pantothenate levels in food, and results correlated highly with results obtained by microbiological assay (Walsh *et al.*, 1980, 1981), the use of radiolabelled tracers is not always desirable in the food context.

A direct, enzyme-linked immunosorbent assay (ELISA) for pantothenic acid has also been developed using specific antibodies active to the vitamin which have been covalently linked to alkaline phosphatase enzyme (Smith *et al.*, 1981). The immobilised phase of the ELISA consisted of a human serum albumin–pantothenate conjugate passively adsorbed to the surface of polystyrene assay tubes. The binding of the enzyme-linked antibody to the pantothenate on the surface of the tubes depends on the amount of free vitamin present initially. The application of ELISA for the determination of pantothenic acid in foods was not reported.

More recently, an indirect ELISA has been developed, particularly directed to food analysis (Morris *et al.*, 1988). Pantothenic acid–bovine serum albumin (BSA) immunogen was synthesised by two different conjugation procedures utilising either ends of the vitamin molecule for conjugation in order to generate antisera of different characteristics. In the first of these, the primary alcohol was derivatised using bromoacetyl bromide to form the bromoacetyl vitamin derivative which was subsequently reacted with reduced and denatured protein (Fig. 3). Radiotracer studies using sodium [1-^{14}C]-pantothenic acid indicated a covalent attachment

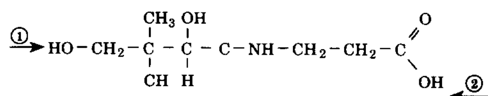

Fig. 3. Coupling of pantothenic acid to protein to use as immunogens for preparation of anti-pantothenic acid antisera: (1) bromoacetyl derivatisation and conjugation; (2) mixed anhydride procedure. [From: Morris, H. C. *et al.* (1988). The development of an enzyme-linked immunosorbent assay (ELISA) for the analysis of pantothenic acid and analogues, Part I—Production of antibodies and establishment of ELISA systems. *J. Micronutr. Anal.*, **4**, 33–45.]

Table 3. Different ELISA formats for the determination of pantothenic acid (PA) and analogues

Compound	Antiserum type[a]	Dilution (v:v)	Solid-phase (PA–KLH) conc. (μg ml^{-1})	Limit detection[b] (μg per well)
Pantothenic acid	BA	1:4 000	0·1	500
Pantothenic acid	MA	1:10 000	1·0	5 000
Pantetheine	MA	1:10 000	1·0	5
Panthenol	MA	1:10 000	1·0	50

[a] BA, Bromoacetyl bromide antiserum; MA, mixed anhydride antiserum.
[b] Calculated by subtracting three standard deviations from the mean zero value.

of the vitamin to the protein and a hapten–protein ratio of 20:1. The second immunogen was prepared by linking pantothenic acid to BSA via the carboxylic acid group by means of the mixed anhydride procedure (Erlanger *et al.*, 1959). A hapten–protein ratio of 17:1 was found by spectrometry for this conjugate. Two corresponding pantothenic acid–keyhole limpet haemocyanin conjugates were synthesised using the above coupling procedures and used to form the ELISA solid phases by passive adsorption to the wells of the microtitration plates.

Both immunogens generated antisera of differing qualities (Table 3). The bromoacetyl antiserum was extremely specific for pantothenic acid with low cross-reactivities for the compounds tested. It is well-known that antibodies exhibit the greatest specificity for portions of the hapten distal to the point of coupling to the protein carrier. This is clearly illustrated in the lack of antibody recognition for pantothenol and pantetheine when the primary alcohol group was used for conjugation, as the differences in structure occur at points furthest away. In contrast, the antibody raised by coupling at the other end of the molecule, i.e. via the carboxylic acid group, showed greater recognition for pantetheine and pantothenol compared with pantothenic acid. Both antisera fail to recognise co-enzyme A, presumably because this contains pantothenic acid derivatised at each end of the molecule.

The characteristics of the two ELISA systems for the determination of pantothenic acid and analogues are given in Table 3. The bromoacetyl antiserum and ELISA system was chosen for the determination of the pantothenate content of a range of foods because of its greater specificity for the free vitamin, lower sensitivities and backgrounds. The ELISA results were compared to those obtained by microbiological assay (Table 4). The foods chosen were representative of the major sources of pantothenic acid in the UK diet and offer a range of food matrices with different interferences. The agreement between the two procedures was generally good, as was the agreement with the food table data. Earlier work also demonstrated that the

Table 4. Results for pantothenic acid content (mg per 100 g) in foods

Food	ELISA	Microbiological assay	Food tables[a]
Milk (whole cow's)	0·36	0·28	0·35
Potato (maincrop, raw)	0·23	0·23	0·30
Bread (white)	0·23	0·23	0·30
Eggs	1·74	1·46	1·80
Liver (lamb's)	8·25	8·65	8·20
Lettuce	0·18	0·07	0·20

[a] McCance and Widdowson's *The Composition of Foods*, Paul and Southgate (1978).

agreement between RIA and microbiological assay for pantothenic acid in a much larger number of foods was good (Walsh *et al.*, 1980, 1981).

During the validation of the ELISA procedure, it was further demonstrated that there was no interference (or matrix effect) with the foods tested. The analysis of different volumes of sample extract gave the correct proportionate responses and the addition of standard amounts of the vitamin to extracts gave satisfactory recovery data.

A comparison of the ELISA with both the microbiological assay and RIA is given in Table 5. Assuming the sample extraction is common to each procedure, then the ELISA can be performed in a working day, whereas the microbiological assay takes 2–3 days. RIA requires a 7 h scintillation counting step which will increase if larger numbers of samples are analysed. RIA also uses more expensive reagents and chemicals but labour costs are comparable to ELISA. However, the major drawback of RIA is the use of radiochemicals which can be a serious limitation to its use in the food laboratory.

Biotin

Biotin is one of the lesser known and most stable of the B-group vitamins. Conventional determination of biotin in food and biological materials is usually by microbiological assay with *L. plantarum*. Biotin can exist in eight different stereoisomers but only one contains vitamin activity, D-biotin. The stereoisomer, L-biotin, is biologically inactive. The organism *L. plantarum* exhibits a growth response to both biotin D-sulphoxide (100%) and D,L-oxybiotin (50%), in addition to that for D-biotin (Bonjour, 1991).

Biotin occurs in food both as the free vitamin and in bound forms where it can be attached to a simple amino acid, for example, as in biocytin, or more complex proteins and peptides. The free vitamin is present in high levels in foods of plant origin. The complete liberation of the bound vitamin requires acid hydrolysis at elevated temperatures (Bonjour, 1991).

In addition to the microbiological assay, isotope dilution assays based on the competition between radiolabelled and unlabelled vitamin for the binding sites on avidin, a naturally occurring biotin binding protein, have been developed. Radiotracers reported include [^{14}]C-biotin (Hood, 1979), [^{3}H]-biotin (Dakshinamurti & Allan, 1979) and [^{125}I]-biotin (Livaniou *et al.*, 1987) derivatives. These assays are sensitive, analysing 1–10 ng biotin, but the application of these techniques for the determination of biotin in food is limited and not conclusive. Although RIA results were reported to agree reasonably well with the microbiological assay for the biotin content in animal feed samples, RIA values were 20–50% higher than the microbiological assay in the case of wheat samples (Hood, 1975).

An ELISA and an EPBA assay have also been developed for the analysis of biotin in food. The EPBA utilises the high affinity of the ureido group of the molecule by avidin. An antibody has been produced for biotin using a biotin–BSA conjugate synthesised through the carboxylic acid group of the side chain (Alcock & Morgan, M.R.A., 1988, pers. comm.) The antibody is extremely specific for D-biotin, and shows no cross-reactivity with dethiobiotin.

The two assays produce slightly different calibration curves for biotin reflecting the differences in the affinity of the vitamin for binding protein on one hand, and the antibody on the other. The curves are shown in

Table 5. Comparison of ELISA, microbiological assay (MA) and RIA for pantothenic acid

Method detail	ELISA	Microbiological assay	RIA
Equipment	Microtitration plate washer and reader, incubator	Autoclave, spectrometer, incubator	Scintillation counter
Reagents	Antisera (primary and secondary), coated plates	Organism, cultures, microinoculum broth, media	Antisera, radiolabelled pantothenic
Assay preparation (h)	2	7	3
Assay length (h)	4	24–36	7
End-method of determination	Absorbance at 450 nm	Turbidity/absorbance at 620 nm counting	Scintillation
Sample throughput	125 sps per week	40 sps per week	100 sps per week
Cost	Fairly low once primary antiserum is available	Reagent costs low but labour high	Reagents/equipment high, labour low

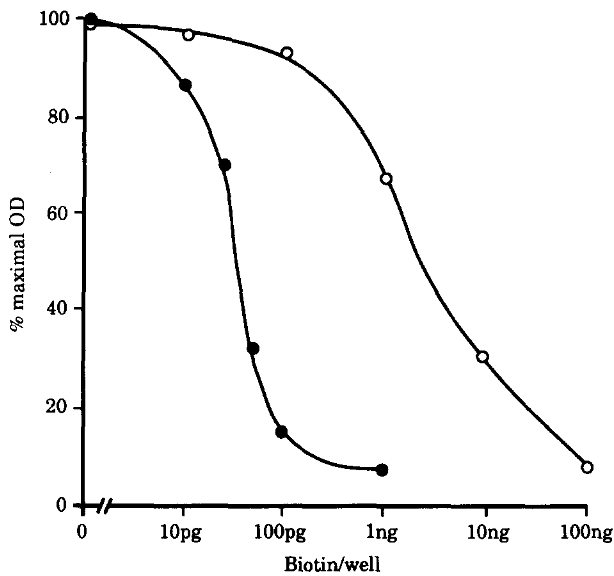

Fig. 4. Biotin standard curves using EPBA (●) and ELISA (○) formats. [From: Lee, H. A. *et al.* (1990). Rapid biospecific methods of vitamin analysis. *J. Micronutrient Analysis*, 7, 261–70.]

Fig. 4. The EPBA is more sensitive, with a limit of detection of 10 μg per well compared to 500 μg per well for the ELISA. The EPBA is likely to give values for the biotin content of foods which are comparable to those obtained by microbiological assay using *L. plantarum*, as it has broad specificity and will recognise other biotin analogues. The EPBA has been used to determine biotin levels in lamb's liver and results agreed favourably with food table data, obtained by microbiological assay (Finglas *et al.*, 1986). Whereas the affinity of avidin for biotin and its analogues is fixed, different antisera preparations could be used to develop a range of highly specific assays for individual biotin analogues.

Vitamin B_6

Vitamin B_6 is found in all foods of both animal and plant origin. It occurs in the free form in foods in small amounts but the major proportion is in the bound form in association with amino acids and proteins (Anderson *et al.*, 1974). The main forms of the vitamin are shown in Fig. 5. Until recently, methods for the determination of vitamin B_6 in foods were based on the microbiological approach, usually with *Saccharomyces uvarum*. This procedure gives a value for the total B_6 content of the food, and is therefore not that useful for nutritional work where levels of the individual forms are needed. In addition, some strains of this organism can give a lower response to the PM form (Gregory 1982).

General problems with the microbiological approach have led to the development of numerous HPLC procedures which are capable of measuring the individual forms (Polansky *et al.*, 1985). However, the initial cost of the equipment and the running costs can be high.

Biospecific methods for vitamin B_6 have entirely made use of the ELISA format. A pyridoxal-BSA conjugate was synthesised through the aldehyde functional group using the sodium borohydride reduction method (Cordoba *et al.*, 1966). This approach was used in an attempt to produce a broad specificity antiserum which would recognise all the B_6 forms (see Fig. 5). The antibodies produced were, however, virtually all specific for PM, although one antiserum produced could recognise PM and PN (Table 6). The preference for the PM form was previously reported in antisera raised to PLP (Cordoba *et al.*, 1966; Vices-Madore *et al.*, 1983) and occurs because the PL is linked to the lysine-amino groups of the protein carrier during conjugation. A cross-reaction was also found for PMP and PL.

An ELISA was set up for PM using R237/B15 and a PL–KLH solid phase conjugate using the same coupling procedure as for the immunogen (Alcock *et al.*, 1990). The assay limit of detection was found to be extremely good (10 μg per well) when compared to other methods of analysis for vitamin B_6. The same authors report the application of ELISA for the determination of PM in a range of foods, and its comparison to the HPLC procedure. The results are given in

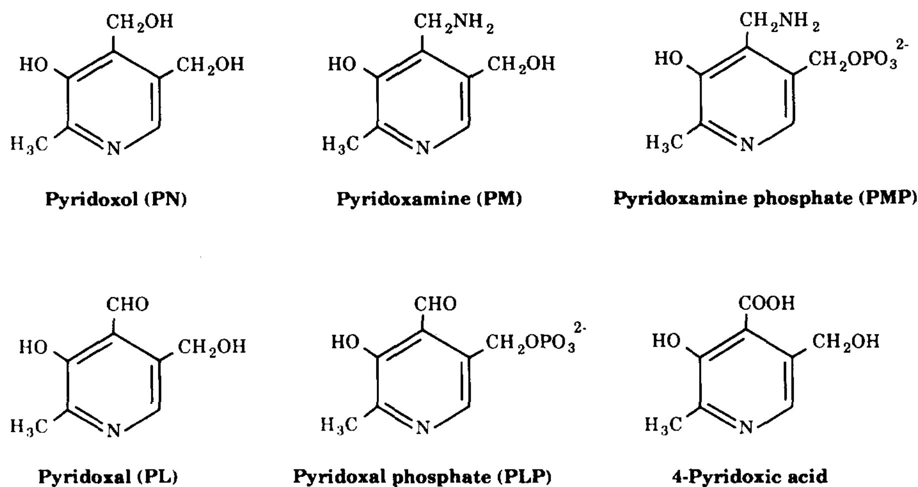

Fig. 5. Structures of vitamin B_6 forms found in food together with 4-pyridoxic acid (a metabolite). [From: Alcock S. C. *et al.* (1990). An enzyme-linked immunosorbent assay for pyridoxamine and its comparison with alternative analytical procedures. *Food and Agricultural Immunology*, 2, 197–204.]

Table 6. % Cross-reaction[a] of various forms of vitamin B_6 for different antisera preparations

Antiserum type	PM	PL	PL	PMP	PLP	4-PA
1	100	<0·1	<0·01	0·8	<0·01	<0·01
2	100	80	9	30	2	2
3	100	26	9	—	—	—
4	100	3	1	—	—	—

[a] Determined by calculating the ratio (as a percentage) of the mass of pyridoxamine required to give 50% displacement from the zero value to the mass of compound of interest required to give 50% displacement.

Table 7. The overall agreement between the ELISA and HPLC procedure was poor, mainly as a result of the much lower PM values found in the chicken leg and beef samples by ELISA compared with the HPLC procedure. This may indicate some problem of interference by the meat-type matrix when assayed by ELISA. The banana sample was also high in PM content but the agreement between the procedures was quite good.

The failure to release the total bound vitamin B_6 content in the food would also lead to erroneously low values in the HPLC procedure. However, antibodies have the ability to recognise both bound and free forms of the vitamin, which gives the potential for the simplification of the extraction procedures.

PM measurement alone by ELISA is of limited use without complementary assays to determine the other forms of the vitamin found in foods. Antiserum R236/B12, which can recognise PN, would be useful for assessing the level of PN in fortified foods as this is the form in which it is added. The preparation of a single antibody that could recognise all B_6 forms irrespective of the functional groups in the C-4 position would seem to be unlikely. A better approach might be to produce specific antisera for each form. Then by mixing the individual antisera, the total B_6 content of the food could be determined. In addition, the individual forms could also be measured using each antiserum. This approach would result in many of the advantages of the HPLC procedure, where all the forms can be measured in a single run, together with assays of greater specificities.

Table 7. Pyridoxamine values (μg per 100 g) of selected foods by ELISA and HPLC

Food type	ELISA	HPLC
Bread (white)	30	38
Milk (whole, cow's)	28	19
Cabbage	9	10
Cucumber	11	14
Potato	75	50
Banana	400	205
White rice	18	10
Brown rice	95	60
Chicken breast	33	15
Chicken leg	85	300
Beef	105	253

Vitamin B_{12}

The term vitamin B_{12} used to describe a group of compounds collectively known as the cobalamins, which are cobalt-containing corrinoids. Cyanocobalamin is actually an artifact of the extraction procedure used where cyanide is added to stabilise the cobalamins present (Chin, 1985). The predominant forms of the cobalamins in food are adensylcobalamin (coenzyme B_{12} and hydroxocobalamin (Farquharson & Adams, 1976). Fortified foods such as breakfast cereals are currently fortified with cyanocobalamin.

The traditional methods of vitamin B_{12} analysis in food are microbiological, usually with either *L. leichnannii* or *Ochromonas malhamensis* as the organism (Chin, 1985). Several workers have found that the latter organism has greater specificity for the cobalamins compared with the former and therefore gives a more accurate assessment of the biological activity of the vitamin (Voigt & Eitenmiller, 1960; Shaw & Bissell, 1960). However, *O. malhamensis* requires a much longer incubation time (3–4 days) compared with *L. leichmannii* and is a drawback.

In clinical assays of vitamin B_{12}, a RIA is commonly used based on the competition of added ^{57}Co-labelled cyanobalamin and free, unlabelled vitamin for the limited number of binding sites provided by the B_{12} binding protein. Intrinsic factor and R-protein have both been used as the binder. Although, RIAs have replaced microbiological procedures for the determination of vitamin B_{12} in blood, a recent report has suggested that the use of commercially available RIA kits for analysis of serum cobalamin may give erroneously high results. This has been attributed to the non-specific binding of the inactive forms (England & Linnell, 1980).

The application of RIA-based kits for the determination of vitamin B_{12} in food has been examined by several workers and has been found to be extraction dependent (Newmark *et al.*, 1976). Higher results were obtained by RIA which was apparently due to the more vigorous extraction procedure used for this technique compared to the microbiological assay (Richardson *et al.*, 1978). Other workers suggest that the presence of cyanide in the extraction procedure gives results similar to the microbiological assay (Marcus *et al.*, 1980).

More recently, an EPBA has been developed for vitamin B_{12} for use with pharmaceutical preparations and vitamin premixes (Tsalta & Meyerhoff, 1987). This approach has been adapted by other workers in the development of a microtitration plate format PBA for the analysis of vitamin B_{12} in fortified foods (Alcock *et al.*, 1992). R-protein was labelled with horseradish peroxidase enzyme (HRP) and the enzyme–protein conjugate further purified by FPLC. In fortified foods, where a single vitamin B_{12} form predominates, e.g. cyanocobalamin, R-protein can be used as it has the advantage of lower costs.

The immobilised phase of the assay was prepared by

coupling cyanocobalamin to KLH using the bromoacetyl bromide procedure (Wyse *et al.*, 1979), i.e. via the primary alcohol group. This was in contrast to previous reports where conjugates were synthesised using the carboxylamide groups on the corrin ring (Olesen *et al.*, 1971; Van de Weil *et al.*, 1974). Improved assay sensitivities were found using the bromoacetyl conjugate compared with the earlier work which allowed the use of the enzyme labelled R-protein at much higher dilutions. The coated microtitration plates and enzyme labelled R-protein solutions were found to be stable for several months without appreciable loss of activity.

The limit of detection of this assay is 9 pg per well and there is a low level of non-specific background binding. The PBA was used to determine the amount of added cyanocobalamin in breakfast cereals. Good agreement was found between the analysed amount determined by EPBA and the theoretical level. Extracts of non-fortified samples were tested for interference in the assay and gave superimposable curves compared with standard solutions. It was also observed that shorter incubation times of about 1 h at 37°C gave similar results to those obtained at 4°C with an overnight incubation.

It was concluded that the EPBA for cyanocobalamin analysis in fortified foods had a number of advantages over both the microbiological assay and RIA. It was easier to perform, had potential for improved reproducibility and much-reduced assay times compared with microbiological procedures. Although the limit of detection for EPBA is sufficient for use with fortified foods, in order to measure natural levels in foods, further improvements in assay sensitivity using alternative end-point detection systems and extraction/cleanup procedures may be required.

Folate

The folates represent an important group of the B-group vitamins. A large number of folate vitamers are known, differing in the extent of the reduction of the pteroyl group, the presence of substituents and the number of glutamyl residues. In order to measure the polyglutamate forms present in biological materials, preliminary enzymatic deconjugation is required.

At present the most widely used and accepted procedure for folates is the microbiological assay using *L. rhamnosus* (ATCC 7469) and *Enterococcus hirae* (ATCC 8043), where a response of the organism to the mixture of folates present in the sample is measured. Even with the introduction of semi-automated microbiological procedures including the microtitration plate format (Newman & Tsai, 1986; Horne & Patterson, 1988), this approach is both time-consuming and demanding in execution. In addition, the response of the organism to the different folate forms is not always identical.

HPLC procedures using both UV and fluorometric detection for the analysis of food folates have been widely reported in recent years. Although adequate resolution for standard mixtures can be obtained, the low folate levels found in most foods can lead to problems in the detection and quantification of the vitamers using this technique. This has been demonstrated in a recent EC interlaboratory comparative study for the determination of folate in Brussels sprouts (Finglas *et al.*, 1993). A group of experienced vitamin laboratories took part using microbiological, HPLC and biospecific procedures. Although the agreement between the microbiological methods was quite encouraging with a between-laboratory CV of about 20%, there was poor agreement between the HPLC procedures, particularly in the types of folate vitamers measured (Table 8).

Several reports in the literature can be found of the analysis of folates in food using RIA, and their comparison to the microbiological assay (Graham *et al.*, 1980; Reingold *et al.*, 1980; Klein & Kuo, 1981). It would appear that the results obtained between the RIA and microbiological procedures are frequently contradictory.

A number of factors are known to affect the folate-binding affinity of the protein used, e.g. temperature, incubation time and particularly pH. In addition, the calibrant used in the assay must be stable and have an affinity for the binding protein that is the same as the folate form found in the samples. Although PGA is more stable than 5-methyl-THF, it should not be used as the assay calibrant in assays run at pH 7·5, because of its greater affinity for the FBP compared to that of 5-methyl-THF at this pH (Givas & Gutcho, 1975). However, at the higher pH of 9·3, PGA is preferred because of its greater stability and equivalent binding compared with 5-methyl-THF. Although the use of commercial RIA kits for the analysis of blood levels of 5-methyl-THF can be justified with careful standardisation of the procedures, the application of these assays to food systems is limited and not conclusive (Gregory, 1985).

In one of the few reports of antibody production to folate compounds, both PGA–BSA and *p*-aminobenzoicglutamic acid–BSA were used as immunogens in rabbits (Ricker & Stollar, 1967). The antiserum obtained from the former, i.e. where the conjugation was through the glutamic acid end of the molecule (Fig. 6), exhibited cross-reactivities not only to PGA (100%) but also to two non-folate analogues (pteroic acid, 71% and 6-aminopterin, 24%). By contrast, where conjugation was through the pteridine ring, as in the second conjugate, even broader specificity antiserum was produced.

As an alternative, an EPBA assay was developed for folate utilising an enzyme labelled folate binding protein from cow's milk (Finglas *et al.*, 1988*b*). The binding protein is commercially available. Standard curves for PGA, 5-formyl-THF and 5-methyl-THF using PGA–KLH coated plates at a concentration of 1 μg ml^{-1} and an enzyme–FBP dilution of 1:100 (v/v) are shown in Fig. 7a. All three curves exhibit good

Table 8. Folate levels (μg per 100 g) in Brussels sprouts by different procedures[a]

Method[b]	Deconjugase enzyme	Mean folate content (number of labs)
Microbiological assay	Human plasma	824 (6)
	Chicken plasma	984 (6)
EPBA	Human plasma	739 (3)
	Chicken plasma	1 320 (2)
RPBA	Human plasma	2 093 (2)
	Chicken plasma	1 290 (2)
HPLC (total)[c]	Human plasma	762 (1)
	Chicken plasma	729 (1)

[a] From Finglas *et al.* (1993).
[b] EPBA: enzyme labelled protein binding assay. RPBA: radiolabelled protein binding assay (commercially available 'radioassay' kits.
[c] Total folate including 5-methyl-THF, 5-formyl-THF and THF forms.

limits of detection of 6, 34 and 36 μg per well, respectively. The relative responses of 5-formyl-THF and 5-methyl-THF are approximately the same and therefore either form could be used as the calibrant in the assay for food work. Although PGA does not occur naturally in foods, it is used for enrichment purposes, so in that case PGA should be used as the calibrant. Other folate forms, with the possible exception of THF, are thought to be unstable during extraction. By changing the plate coating conjugate from PGA–KLH to 5-formyl-THF the response of the PGA folate form can be modified and becomes closer to the response of the other two folate forms tested (Fig. 7b).

The EPBA was used for the determination of folate in a range of raw and cooked vegetables (Finglas *et al.*, 1988*b*). Samples were also analysed by microbiological assay and the results from the two assays compared. Good agreement was obtained between the methods with a linear relationship over the range 0–400 μg per 100 g found. The EPBA could be carried out in one working day compared to 2–3 days for the microbiological assay.

Results from the recent interlaboratory comparison of folate methods (Finglas *et al.*, 1993) are given in Table 8. The EPBAs used were essentially based on the same format as described above but the results between laboratories varied from 378 to 1051 μg per 100 g (n = 3) for human plasma enzyme and from 903 to 1601 μg per 100 g (n = 2) for chicken pancreas deconjugase enzyme. The HPLC procedures indicated the major folate forms present in Brussels spouts were 5-methyl-THF (50%), THF (30%) and 5-formyl-THF (20%). Two of the laboratories used 5-methyl-THF as the calibrant, whereas the third used PGA.

The study concluded that the main limitation of both the EPBA and RIA is the response of the indi-

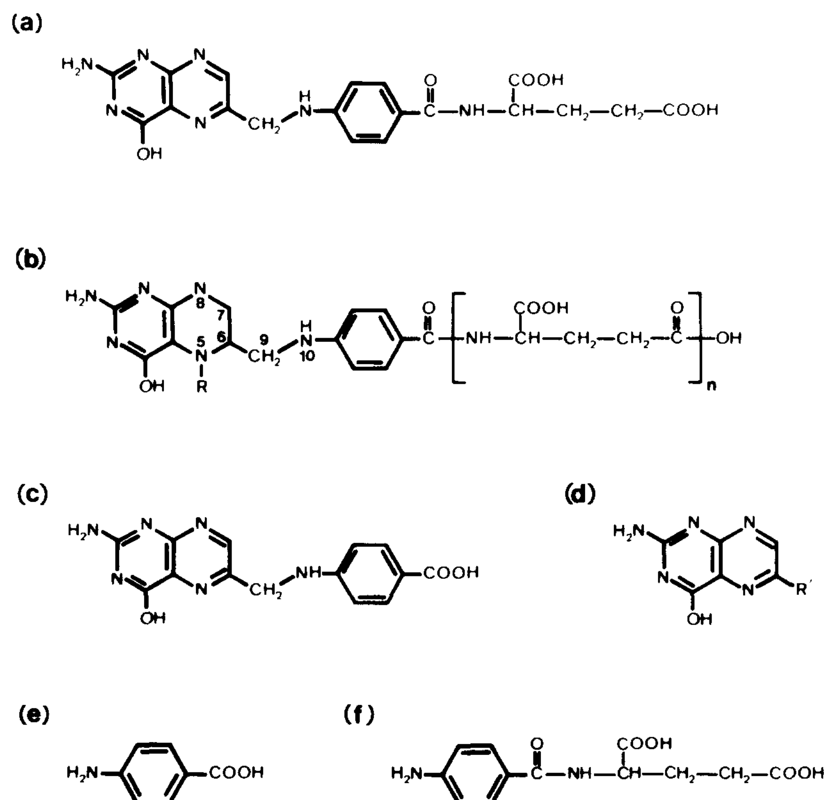

Fig. 6. Folate vitamins and structurally related compounds: (a) folic or pteroylglutamic acid; (b) 5,6,7,8-tetrahydrofolic acid (R = H), 5-formyltetrahydrofolic acid (R = CHO), 5-methyltetrahydrofolic acid (R = CH_3); (c) pteroic acid; (d) pterine (R' = H), pterin-6-carboxylic acid (R' = COOH); (e) p-aminobenzoic acid (PABA) and (f) *N*-(*p*-aminobenzoyl)-L-glutamic acid (PABGA). [From: Finglas, P. M. *et al.* (1988). The development and characterisation of a protein-biding assay for the determination of folate—potential use in food analysis. *J. Micronutrient Analysis*, **4**, 295308.]

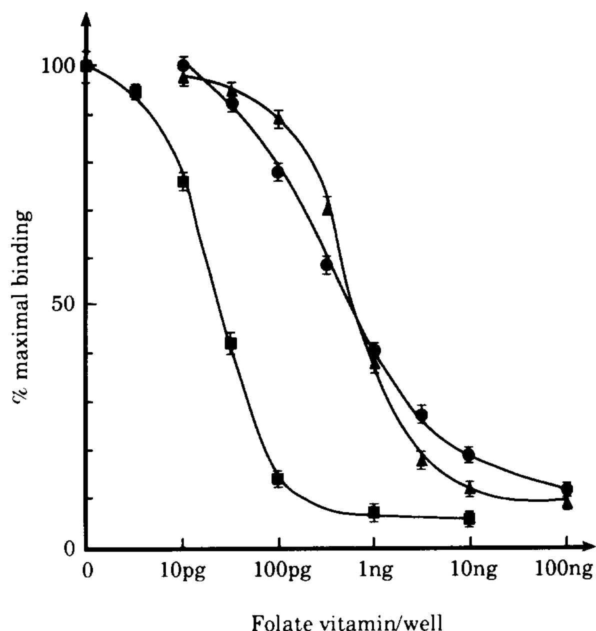

Fig. 7a. EPBA standard curves for various folates using PGA–KLH plates coated at 1 μg ml^{-1} and FBP–enzyme conjugate at 1:100 (v:v). ■, PGA; ●, 5-CHOTHF; ▲, 5-CH_3THF. Marker bars indicate ±1 SD. [From: as for Fig. 6.]

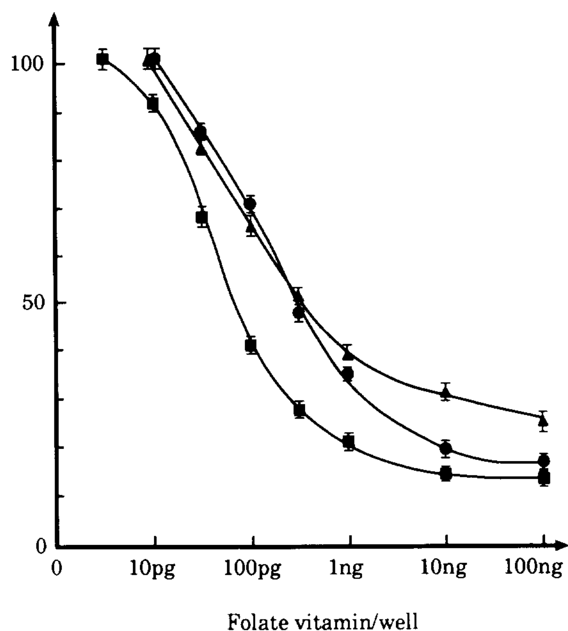

Fig. 7b. EPBA curves for various folates using 5-formyltetrahydrofolic acid–KLH plates coated at 10 μg ml^{-1}. ■, PGA; ●, 5-CHOTHF; ▲, 5-CH_3THF. Marker bars indicate ± 1 SD. [From: as for Fig. 6.]

vidual folate forms to the FBP used. Careful control of the pH of the assay buffer used and choice of the assay calibrant is needed, if these assays are to be applied to the measurement of food folates.

The EPBA assay for folate offers the potential for broad specificity and sufficient sensitivity for the measurement of food folates. Further work, however, is needed to standardise the methodology, particularly in the choice of calibrant and assay conditions, before results can be confidently compared with other procedures such as the microbiological assay.

CONCLUSIONS

Biospecific methods of analysis offer the analyst considerable potential to fulfil the increasing need for more rapid, sensitive and specific vitamin assays. The techniques do not require highly skilled operators or sophisticated detection systems and can be easily automated.

EPBAs are useful for assays of broad specificity where the analysis of a group of vitamers is required, as in the case of folate or biotin. Thus, the results obtained with these assays may be expected to correlate better with the results of microbiological procedures.

However, the ELISA format using antibodies has the greater potential for the detection of specific vitamin forms, which can be useful for nutritional studies including the assessment of vitamin bioavailability in food and the measurement of vitamin status. The specificity of antibodies means that they could be used in conjunction with chromatographic procedures to assist in the identification/quantification of individual vitamers because normal detection systems are inadequate. In addition, by the careful selection of immunogen conjugates, or by mixing two or more different antisera together, antibodies could be used to detect selected combinations of vitamers.

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