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An in vitro method for estimating biologically available vitamin B_6 in processed foods

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1. An in vitro method which used enzymic digestion of the food matrix to release biologically available vitamin B_6 is described.

2. Vitamin B_6-fortified liquid model foods were thermally processed. After these foods had been freeze-dried, one part was subjected to enzymic hydrolysis at pH 2·0 with pepsin (*EC* 3.4.23.1) followed by a hydrolysis at pH 8·0 with pancreatin. The vitamins that were found in the supernatant fraction, after an acidified methanol treatment of the hydrolysate, were estimated by high-performance liquid chromatography (HPLC). The other part was given to rats who were kept on a vitamin B_6-depleted diet.

3. The biologically available vitamin B_6 content of the processed model foods, as determined by rat bioassay, showed good correlation with the vitamin B_6 determined by HPLC.

4. It has proved possible to use this in vitro, two-stage enzymic digestion system followed by HPLC determination to determine biologically available vitamin B_6 in vitamin B_6-fortified processed model foods.

Routine estimates of biologically available vitamin B_6 in foods cannot be carried out by animal bioassay methods because of the long period required for such a study and the heavy expenditure that it incurs. It is possible that, because of these factors, thermal processes for food preservation do not take into account the effect of such processes on the biological availability of micronutrients such as vitamin B_6.

The in vitro method described here has the advantages of low cost and short analysis time and therefore the potential for wide use as a screening technique for biologically available vitamin B_6 in foods undergoing thermal processing.

METHODS

Casein (vitamin-free), Alphacell (non-nutritive bulk), maize oil, vitamin mix (AIN-(76); vitamin B_6 omitted and fortified with 50 mg vitamin K/kg mix), choline chloride and mineral mix AIN-(76) were obtained from ICN, Nutritional Biochemicals, Cleveland, OH. Maize-syrup solids (dextrose equivalent 12) and waxy maize starch were obtained from American Maize Products Co., Hammond, IN, and Anheuser-Busch Industrial Products, Lafayette, IN respectively. Ray-sorb Erlenmeyer flasks were obtained from American Scientific Products, McGaw Park, IL. Pepsin (porcine; 1:10000; *EC* 3.4.23.1), pancreatin (porcine; 4 × National Formulary), pyridoxal hydrochloride, pyridoxamine dihydrochloride and pyridoxine hydrochloride were obtained from Sigma Chemical Co., St Louis, MO. Male, weanling (21-d-old), Sprague Dawley rats were obtained from Harlan Industries, Indianapolis, IN.

Model food preparation

Model food slurries were formulated as shown in Table 1 and weighed amounts introduced into metal cans. In the experiments with pyridoxine (PN) these slurries were fortified to 20, 60 and 100% of the US recommended daily allowance levels for vitamin B_6 ((US) National Research Council, 1980) based on the requirement for humans, i.e. for 65 g protein in the model food slurry, 2·2 mg PN were added at the 100% level of fortification.

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Table 1. *Composition of model foods (g/kg)*

Casein (vitamin-free)	35
Maize-syrup solids	111
Waxy maize starch	37
Alphacell	10
Sodium chloride	7
Water	800

Model foods with pyridoxine were fortified at levels of 20, 60 and 100% of the US recommended daily allowance (National Research Council, 1980) while model foods with pyridoxamine and pyridoxal were fortified at 20 and 100% levels of the US recommended daily allowance for humans, based on the daily protein requirement.

Table 2. *Composition of diets for rat bioassay (g/kg)*

Diet...	Depletion	Control	Experimental
Casein (vitamin-free)	198·0	189·0	189·0
DL-Methionine	2·0	2·0	2·0
Sucrose	605·0	568·0	568·0
Alphacell	93·0	90·5	90·5
AIN-(76) salt mix*	40·0	38·2	38·2
AIN-(76) vitamin mix*†	12·0	12·0	12·0
Maize oil	50·0	50·0	50·0
Model food	0·0	50·0 (unfortified)	50·0 (fortified)

* ICN, Nutritional Biochemicals.
† Vitamin B_6 omitted and fortified with 50 mg vitamin K/kg mix.

In the experiments with pyridoxamine (PM) and pyridoxal (PL), the model food slurries were fortified to 20 and 100% of the vitamin B_6 requirement. Model food slurries without any fortification were taken to make the model food component of the control diets.

After filling in the previously described manner, the cans were sealed and thermally processed at 116° for 50 min and cooled to room temperature immediately afterwards. The contents were then freeze-dried and powdered in a blender before analysis by either rat bioassay or enzymic hydrolysis.

Rat bioassay

Biologically available vitamin B_6 in the processed, freeze-dried model foods was estimated by a rat growth assay. Male, weanling (21-d-old), Sprague Dawley rats (40–50 g weight range) were weighed on arrival in the laboratory and assigned to groups of six. They were housed individually in cages with wire bottoms to minimize coprophagy. The vitamin B_6 depletion diet (Table 2) and water were provided *ad lib* for 2 weeks. At the end of this depletion period, the control and experimental diets (Table 2) were substituted for the vitamin B_6 depletion diet. Weekly weighings of the animals were carried out for the next 3 weeks. The food consumed was recorded twice weekly at which time the spilled food was also weighed. Utmost care was taken in the preparation of the diets to minimize exposure to light.

Six animals per control and experimental groups were used for assessment of biological availability. In all studies, the control groups were given one of the pure vitamins (PL, PM or PN) mixed with the control diet at 0·0, 0·25 and 0·50 mg/kg diet. These vitamin standards were prepared by grinding the equivalent of 0·100 g vitamin (free base) with 99·900 g sucrose in a ball mill for 8–10 h. The homogeneity of these standards was tested by high-performance liquid chromatography (HPLC) analysis of three random samples taken from each mix.

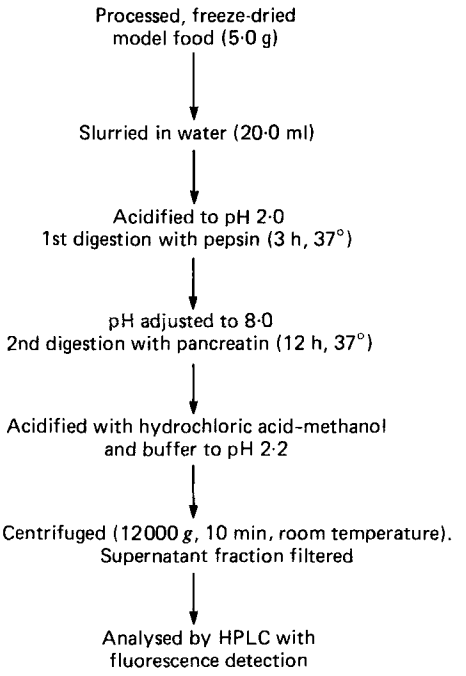

Fig. 1. Scheme for in vitro digestion of a model food and analysis by high-performance liquid chromatography (HPLC).

Using the feed conversion efficiency (weight gain/food consumed) values from the control groups, standard linear regressions were obtained for the three vitamins (Table 3, p. 239). From these values, the vitamin contents in the experimental diets were obtained. Plots of vitamin contents thus obtained *v.* the level of fortification were used to obtain the linear regressions (Table 3, p. 239). These curves were used in the comparison with the values from the in vitro method.

In vitro enzymic digestion method

An enzymic digestion method (Fig. 1) was developed to quantify available vitamins. A 5 g portion of each powdered model food, in triplicate, was weighed accurately and introduced into a Ray-sorb Erlenmeyer flask wrapped with aluminium foil. Distilled water, 20 ml, was added and the food slurried. This slurry was acidified to pH 2·0 with a known amount of 1·5 M-hydrochloric acid. With the introduction of 5·0 mg pepsin, the first digestion step was begun. The flasks were swirled to ensure mixing, then fixed to the extended arm of a wrist-action shaker (Burrel Corporation, Pittsburg, PA), and incubated in a water-bath at 37° with shaking for 3 h. At the end of this period, a predetermined amount of 1 M-sodium hydroxide was added to adjust the pH to 8·0 and the second digestion step begun with the addition of 3·5 mg pancreatin suspended in 2·0 ml 1·0 M-phosphate buffer, pH 8·0. The incubation was continued for a further 12 h period.

The period of the pancreatin digestion was selected to give optimum release of the vitamins. To verify this, a preliminary experiment was conducted in which model foods with the highest level of fortification were put through the digestion process with samples being analysed for the vitamin content every 3 h (Fig. 2).

After the second stage of the digestion, 5·0 ml portions were pipetted into pre-matched centrifuge tubes containing 2·5 ml acidified methanol (approximately 0·12 M with respect

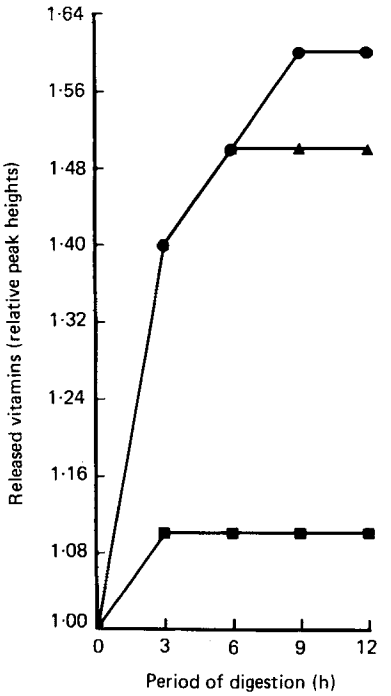

Fig. 2. Release of vitamins from pancreatin digestion of processed model foods: (●), pyridoxal; (■), pyridoxamine; (▲), pyridoxine.

to HCl). This adjusted the pH to about 2·2. A further 2·5 ml 0·1 M-phosphate buffer, pH 2·2, was added to the tubes and they were centrifuged at 12000 *g* for 10 min at room temperature. The clear supernatant fractions were removed with hypodermic syringes and filtered through 0·45 μm nylon 66 membranes (Rainin Instrument Co. Inc., Woburn, MA) before analysis. To determine the recovery of vitamin B_6 from the extraction procedure, 1·0, 0·5 and 0·5 ml of standard PL, PM and PN solutions respectively were added to flasks containing unfortified processed model foods. The amount remaining after the enzymic digestion was determined by HPLC. Triplicate determinations were carried out for each vitamin. All chemical manipulations were carried out in the dark.

HPLC analysis

HPLC analysis was performed by a method similar to that of Gregory & Kirk (1978). An HPLC system (Waters Associates, Milford, MA), equipped with a M-6000A pump, a U6K septumless injector and a μ-Bondapak C-18 column was used. The eluent was monitored by a fluorescence spectrophotometer (Model 650–10S; Perkin Elmer Corporation, Norwalk, CT) equipped with a 15 μl flow cell. The excitation monochromator was set at 295 nm (slit width 10 nm) and the emission monochromator at 405 nm (slit width 10 nm). The mobile phase for the isocratic separation consisted of 0·035 M-potassium dihydrogen phosphate acidified to pH 2·2 with phosphoric acid to which methanol was added to a level of 30 ml/l. This was pumped at 2·0 ml/min at a back pressure of approximately 12400 kPa.

Standard vitamin solutions were prepared by dissolving the equivalent of 12·5 mg vitamin-free base (i.e. 15·2 mg pyridoxal hydrochloride, 17·9 mg pyridoxamine dihydrochloride or 15·2 mg pyridoxine hydrochloride) in 100 ml water and diluting portions of this solution with phosphate buffer (pH 2·2) to obtain the final standards. A standard curve

Table 3. *Linear regression determinants for the in vivo method for determining biological availability of vitamin B_6: (a) vitamin content in control diet* v. *feed conversion efficiency, (b) level of fortification* v. *biologically available vitamin B_6 contents of the experimental diets*

(Mean values with their standard errors for six animals per group)

Vitamin		Slope		Intercept		Correlation coefficient	Coefficient of variation (%)
		Mean	SE	Mean	SE		
Pyridoxal	(a)§	0·3411	0·0394	+0·098¶	0·01	+0·96	13
	(b)†	0·0042*	0·0004	−0·047NS	0·03	+0·96	28
Pyridoxamine	(a)‖	0·3263	0·0462	+0·113¶	0·01	+0·85	24
	(b)†	0·0031*	0·0007	+0·028NS	0·05	+0·80	47
Pyridoxine	(a)§	0·3277	0·0274	+0·126¶	0·01	+0·97	11
	(b)‡	0·0032*	0·0006	+0·051NS	0·04	+0·82	33

NS, not significantly different from zero.

* Significantly different from zero ($P \leqslant 0·05$).

† Two levels of fortification (20 and 100%) of US recommended daily allowance ((US) National Research Council, 1980).

‡ Three levels of fortification (20, 60 and 100%) of US recommended daily allowance ((US) National Research Council, 1980).

§ Three levels: 0·00, 0·250 and 0·500 mg pyridoxal or pyridoxine (free base)/kg diet.

‖ Three levels: 0·00, 0·233 and 0·466 mg pyridoxamine (free base)/kg diet.

¶ Feed conversion efficiency at 0·00 mg vitamin B_6/kg diet.

relating peak height to the amount of vitamins in a 10 μl injection was calculated by linear regression methods for each vitamin (Table 4, p. 240).

The amounts of vitamins released on enzymic digestion of the model foods were determined using the HPLC standard curves and the values obtained plotted against the level of fortification to obtain three lines for the three vitamins (Table 4). These curves were used to compare the biologically available vitamin content as determined by the animal study and the in vitro method.

To confirm the identity of the compound eluting with the same retention time as that of PM in the PL-fortified samples and also in the PL-added samples, thin-layer chromatography was performed on the filtered, concentrated extracts. Silica gel G layers were used as the stationary phase while the mobile phase was *n*-butanol–pyridine–acetic acid–water (15:10:3:12, by vol.) (Polyanovaskii, 1963). Spots were visualized by spraying the dried plates with a 2,6-dichloroquinone chlorimide solution (1 g/l absolute ethanol) and exposing the plates to ammonia vapour.

Statistical analysis

The two sets of curves obtained by plots of level of fortification *v.* biologically available vitamin content from the rat bioassay (Table 3) and the in vitro method (Table 4) were compared to determine their similarity. Due to the dissimilar variances of the two sets of regression curves, the method of Ostle (1963) was used to cover this comparison.

RESULTS

Table 3 shows that the coefficients of variation of the rat bioassay values for PL and PM were higher than that for PN for the control diets. This same trend, however, was not evident in the values from the experimental diets. Overall the variation was much higher in the experiments with PM as compared with PL and PN.

Table 4. *Linear regression determinants for the in vitro method for determining the biological availability of vitamin B_6: (a) standards from high-performance liquid chromatography, (b) level of fortification* v. *biologically available vitamin B_6 contents of the experimental diets*

(Mean values with their standard errors for triplicate determinations)

Vitamin		Slope		Intercept		Correlation coefficient	Coefficient of variation (%)
		Mean	SE	Mean	SE		
Pyridoxal	(a)	55·35†	0·03	—	—	+0·999	2
	(b)‡	0·00374*	0·00002	−0·006NS	0·001	+0·999	1
Pyridoxamine	(a)	266·08†	0·07	—	—	+0·999	1
	(b)‡	0·00318*	0·00006	+0·005NS	0·005	+0·998	4
Pyridoxine	(a)	68·74†	0·06	—	—	+0·999	3
	(b)§	0·00332*	0·0001	+0·008NS	0·007	+0·989	6

NS, not significantly different from zero.
* Significantly different from zero ($P \leqslant 0{\cdot}05$).
† Peak height (mm)/vitamin content (ng) in an injection volume (10 μl).
‡ Two levels of fortification (20 and 100%) of US recommended daily allowance ((US) National Research Council, 1980).
§ Three levels of fortification (20, 60 and 100%) of the US recommended daily allowance ((US) National Research Council, 1980).

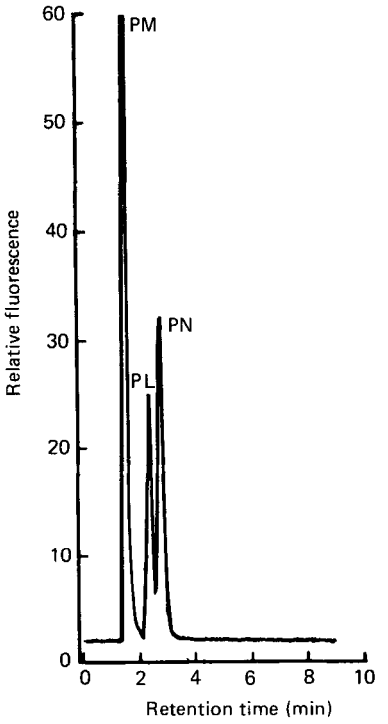

Fig. 3. Chromatogram of the vitamin B_6 standards: PL, pyridoxal; PM, pyridoxamine; PN, pyridoxine.

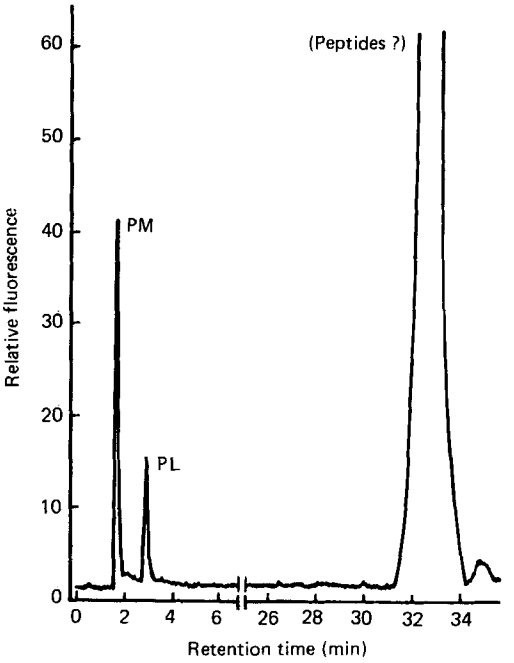

Fig. 4. Typical chromatogram of a digested model food (pyridoxal fortified).

Compared with the very high coefficients of variation in the rat bioassay values, the HPLC values were much more accurate (Table 4).

HPLC separation of the vitamin standards was achieved in less than 4 min (Fig. 3). There was no interference from any food components in the analysis of the processed food samples. The closest components eluting after the vitamin B_6 compounds, took over 20 min to elute after the peaks of interest (Fig. 4).

When the regression lines for the level of fortification *v.* available vitamin contents of the experimental diets for each vitamin, obtained by either rat bioassay (Table 3) or the in vitro method (Table 4) were compared statistically by the method of Ostle (1963), no significant difference was found between each pair.

DISCUSSION

Of the criteria used to assess biological availability of vitamin B_6 in foods, feed conversion efficiency (weight gain/food consumed) has been consistently well correlated with other assays (Nguyen *et al.* 1981; Gregory, 1980*a*,*b*) or has given better correlations compared with an enzymic index like aspartate aminotransferase (*EC* 2.6.1.1) activity (Yen *et al.* 1976; Gregory & Kirk, 1978; Gregory & Kirk, 1981*a*). Especially where purely synthetic diets are used, feed conversion efficiency values should suffice for biological availability estimates.

PL and PM are comparatively unstable to light, unlike PN (Saidi & Warthesen, 1982), and this fact may help to explain the higher variation of the rat bioassay values associated with PL and PM for the control diets (Table 3). This was evident in a preliminary study where a series of diets with differing levels of PM, PL and PN were prepared without any precautionary measures to prevent exposure to light. Analysis of these diets after a period

of 5 weeks showed that the PM and PL levels of all the diets were reduced drastically while the PN levels remained unaltered.

The work of Mehansho *et al.* (1979), Hamm *et al.* (1979) and Buss *et al.* (1980) has shown that the rate of transport of the B_6 vitamins was similar in all parts of the small intestine and also that PL was removed from the intestinal lumen faster than either PM or PN. The feed conversion efficiencies found in the present study seem to show this same trend in terms of the higher biological activity of PL compared with that of PM and PN (Table 3).

PL, PM and their phosphates are the prosthetic groups for transaminases and are known to be tightly but noncovalently linked to the enzyme protein (Lehninger, 1975) in tissue. Yasumoto *et al.* (1977) reported that PN in rice bran and wheat germ is associated with the starch fractions. It is therefore postulated that enzymic hydrolysis of the polymeric food matrix would release biologically available vitamin B_6.

The in vitro method of analysis presented no major chemical problems unlike those experienced in in vitro mineral availability studies where pH-dependent solubility (Miller *et al.* 1981) and the ability to exchange with ions in intestinal tissue (Wein & Schwartz, 1983) complicated the interpretation of results.

It proved very helpful to carry out blank digestions in duplicate to determine the amounts of acid and alkali that had to be added. In earlier in vitro studies where protein digestibility (Akeson & Stahman, 1964) and iron biological availability (Rao & Prabavathi, 1978; Nelson & Potter, 1980) were determined, a microbial inhibitor was added before the pancreatin digestion. Such agents interfered with the HPLC analysis and proved to be of no benefit in view of the shorter digestion time (12 h *v.* 24 h) and were therefore avoided. Alternatively, the concentration of the phosphate buffer, pH 8·0, was kept at about 1·0 M, which limited the fall in pH after 12 h to about one unit. Microbial contamination was not observed as evidenced by the absence of colonies on tryptone soya agar (Oxoid Ltd., Columbia, MD) plates inoculated with the digests.

Preliminary experiments did indicate that the maximum release of the vitamins occurred after about 9 h of digestion with pancreatin (Fig. 2). However, the 12 h period was selected due to the convenience it offered in being able to start an analysis at the end of 1 d, digest overnight and complete the HPLC work the next day.

Acidified methanol proved to be a very efficient precipitant for the model food digests. With trichloroacetic acid the precipitation was incomplete, leading to problems in sample filtration.

Recoveries from the work up procedures were about 100% for PM and PN. PL, however, had lower but consistent recoveries (Table 5). Transamination of PL occurred during thermal processing of model foods as well as during enzymic digestion. This was confirmed by the appearance of a blue spot, with R_f similar to that of PM, on the thin-layer chromatogram of the digested extracts of the foods fortified with PL. This formation was also observed in the extracts from unfortified foods where PL was added just before digestion. The conditions used for processing and digesting seem to be very favourable to transamination of PL to PM, which is known to occur readily in the presence of amino acids (Snell, 1958, 1981). When calculating recoveries and applying consequent correction factors, this transamination was taken into account. Assessment of the degree of transamination due to both thermal processing and enzymic digestion was done by the addition of a known volume of a standard solution of PL to an unfortified model food and determining the extent of PM formation after both processing and digesting.

Many methods, including turbidimetry and fluorescence spectroscopy, have been used for vitamin B_6 analysis (Gregory & Kirk, 1981*a*). Among these, HPLC methods using reversed polarity stationary phases have shown the greatest promise (Gregory & Kirk, 1978; Schrijver *et al.* 1981). The retention times of PM, PL and PN (Fig. 2) proved adequate for

Table 5. *Vitamin recovery from the in vitro method*
(Mean values and standard deviations for four determinations per vitamin)

Vitamin	Recovery (%) Mean	SD
Pyridoxal	67·2	6·5
Pyridoxamine	125·8	8·8
Pyridoxine	107·8	1·3

Table 6. *Comparison of methods for estimating vitamin B_6*

	Rat bioassay			In vitro method		
	Slope		Coefficient of variation	Slope		Coefficient of variation
Vitamin	Mean	SE	(%)	Mean	SE	(%)
Pyridoxal	0·0042	0·0004	28	0·00374	0·00002	1
Pyridoxamine	0·0031	0·0007	47	0·00318	0·00006	4
Pyridoxine	0·0032	0·0006	33	0·00332	0·0001	6

good resolution. The only other fluorescent components which eluted from the column about 20 min later were probably fluorescent peptides (Teale & Weber, 1957) containing aromatic amino acids.

These late eluting fluorescent peptides (Fig. 4) limit the number of analyses to two samples per hour. However, the supernatant fraction after centrifuging could be purified using a Sep-pak C_{18} cartridge (Water Associates), whereby the late eluting compounds could be separated from the vitamins before HPLC analysis (Ekanayake, 1983) thus more than doubling the number of analyses that could be performed.

When the pairs of regression curves for the level of fortification *v.* biologically available vitamin content for each vitamin were compared statistically (see summary Table 6), no significant difference was found between each pair. This would imply that the available vitamin B_6 in the model foods for all three vitamins as measured by the rat bioassay and the in vitro method showed no difference.

Also, from the coefficients of variation, it is apparent that the determinations done by the in vitro method are far more accurate than the values from the bioassay, i.e. coefficients of variation are one order more in the rat bioassay. This limitation with animal bioassays for biological availability measurement of vitamin B_6 has been reported by other workers (Woodring & Storvick 1960; Gregory & Kirk 1981*b*) as well.

Compared with animal bioassay methods, the reported in vitro method for determining biologically available vitamin B_6 in processed food has the advantages of greater accuracy, far shorter analysis time, much lower cost per analysis and the versatility of application to a range of foods (Ekanayake, 1983).

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