Reliable and Sensitive High-Performance Liquid Chromatographic Method with Fluorometric Detection for the Analysis of Vitamin B-6 in Foods and Feeds

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A high-performance liquid chromatographic (HPLC) method with fluorometric detection is described for the analysis of vitamin B-6 in foods and feeds. A simple extraction of the sample with a 5% trichloroacetic acid solution was found to provide complete extraction. The phosphate group was enzymatically removed from the phosphorylated vitamers to facilitate HPLC analysis. The unphosphorylated B-6 vitamers were fully separated by paired-ion reversed-phase (C_{18}) HPLC within 35 min and detected by fluorometry. 4-Deoxypyridoxine was used as internal standard. High recovery and precision were obtained. The range was from 0.02 to 25 μg/g. Varying the composition of the mobile phase changed the capacity factors of the vitamers, an effect which can be used to deal with interferences. The vitamin B-6 content of several foods and feeds was determined with the developed HPLC method and the classical microbiological method using *Saccharomyces uvarum*. The higher contents measured by HPLC (average difference 40%) could be explained for the greater part by systematic errors in the microbiological method. The results obtained indicate that the described HPLC procedure can serve as a simple and reliable method for the analysis of vitamin B-6 in foods and feeds.

Keywords: *Vitamin B-6, pyridoxal, pyridoxine, pyridoxamine, HPLC, fluorometry, foods, feeds, enzymatic hydrolysis, microbiological method*

INTRODUCTION

The determination of vitamin B-6 in foods, feeds, and other biological materials is complicated by the existence of six forms of the vitamin. These are pyridoxal, pyridoxine, and pyridoxamine and their 5′-phosphate esters. Moreover, vitamin B-6 may be bound to food components (Kabir et al., 1983a) and may undergo hydroxylation in the sample (Snell, 1981).

The classical method for the analysis of this vitamin is the microbiological assay using *Saccharomyces uvarum* (ATCC 9080). This method has several drawbacks. It is very time-consuming and susceptible to artifacts. Moreover, there is a difference in growth response of the yeast used to the different forms of vitamin B-6 (Gregory, 1988) or to the presence of vitamin B-6 β-D-glucosides in plant-derived samples. The response to pyridoxamine 5′-phosphate (PMP) is approximately 5% of the response of pyridoxine (PN) and pyridoxal (PL). In addition, a microbiological assay like the one used for vitamin B-6 has a high coefficient of variation.

Therefore, it is not surprising that alternative methods have been developed for the quantification of vitamin B-6. Several different HPLC methods have been described for the analysis of the individual forms of vitamin B-6. Due to the existence of multiple biologically active forms of the vitamin and its low level in complex matrices, the HPLC determinations that have been described are still troublesome as reviewed by Gregory (1991).

This prompted us to optimize the HPLC method, by modification and adaptation of methods previously described. As already mentioned, one of the problems of vitamin B-6 analyses is the existence of several vitamers. With respect to the use of the results of vitamin B-6 analyses, for example, for food composition tables, it suffices, in our opinion, to know the total amount of vitamin B-6 calculated from the individual contents of pyridoxal, pyridoxine, and pyridoxamine, irrespective of whether they exist in their free or phosphorylated form. With a proper hydrolysis of the 5′-phosphate esters, only three structurally closely related forms have to be analyzed. This means that the development of a method for the quantification of vitamin B-6 can be divided into (i) proper extraction method for the several vitamers, (ii) hydrolysis of the phosphorylated and glycosylated forms, and (iii) HPLC separation and detection of the three unphosphorylated B-6 vitamers. Our aim was to develop a method that is flexible enough to be applied to all kinds of foods and feeds.

MATERIALS AND METHODS

Apparatus. HPLC was performed using a Gilson constant-flow pump (Meyvis, Bergen op Zoom, Netherlands), an ISS-100 automatic injector (Perkin-Elmer, Gouda, Netherlands), and a Shimadzu Type RF-530 fluorescence spectrophotometer (Pleuger & Lameris, Amstelveen, Netherlands) with a 100-μL Hellma flow cell (Hellma Benelux, The Hague, Netherlands). A Knauer stainless-steel column (12.5 cm × 4.6 mm i.d.) was laboratory-packed with ODS Hypersil 3 μm (Shandon Southern Products, Astmoor, U.K.) by the balanced-density slurry technique, using a Haskel Type DSTV 150 pump (Amman Technik, Stuttgart, Germany) at a nitrogen pressure of 60 MPa. The slurry and packing solvents were 2-propanol and methanol, respectively. A four-channel peristaltic pump (Whatson & Marlow, Falmouth, U.K.) was used for postcolumn reagent supply. Elution profiles were displayed on a Kipp BD 41 recorder (Kipp Analytica, Delft, Netherlands).

Reagents. Pyridoxal (PL), pyridoxine (PN), pyridoxamine (PM), and 4′-deoxypyridoxine (DOP) were obtained as their hydrochloride forms from Sigma Chemical Co. (St. Louis, MO). Pyridoxal 5′-phosphate (PLP) and pyridoxamine 5′-phosphate (PMP) were also obtained from Sigma.

Stock solutions were prepared in distilled water with a few drops of phosphoric acid at an approximate concentration of 100 μg/mL each. These solutions were stored in the dark at 4 °C and were stable for at least 2 months. Taka-Diastase was obtained from Pfalz and Bauer (Waterbury, CT) and β-glucosidase from Sigma.

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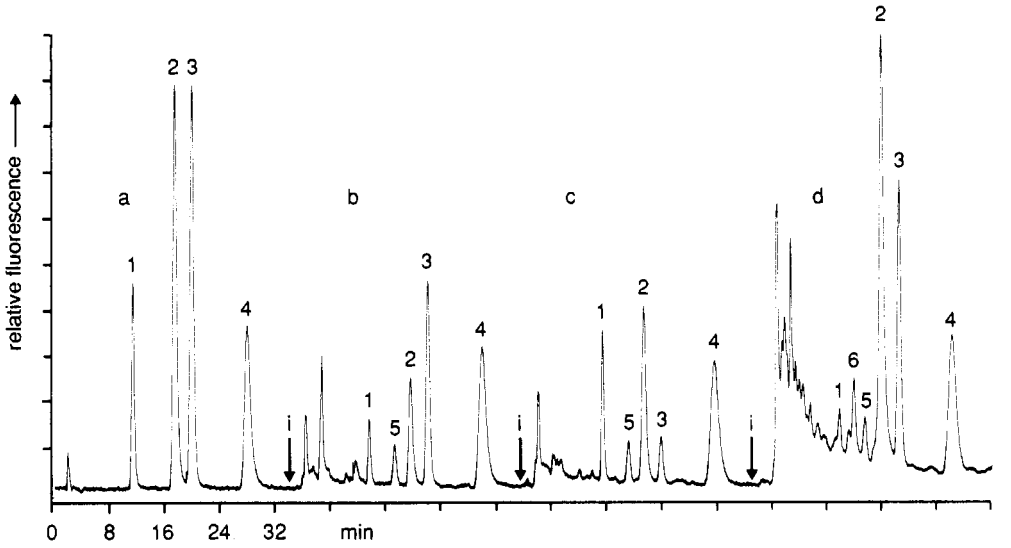

Figure 1. Typical elution profiles of the working standard solution (a) with (1) PL, (2) PN, (3) PM, and (4) DOP and of extracts of banana (b), chicken breast (c), and Brussels sprouts (d). i, moment of injection; 5, unknown peak; 6, probably PN 5′-β-D-glucoside.

The pairing-ion 1-octanesulfonic acid was purchased from Waters Millipore (Waters Associates, Etten-Leur, Netherlands) as a ready-to-use reagent (Pic B8). All other chemicals were of analytical grade.

Sample Handling and Storage. Food samples were obtained from local stores. After grinding and homogenization at 4 °C, some samples were kept frozen (−20 °C) for several months. It was found that this storage did not result in an appreciable loss of B-6 vitamers (data not shown). Meat samples were frozen and cut into small pieces before they were blended with a Waring blender, Type 91-358 (New Hartford, CT) under liquid nitrogen. The homogenized meat samples were stored in a freezer at −20 °C until analysis. All frozen samples were thawed in a water bath at room temperature until they were semiliquid, mixed by stirring, and weighed into the bottle.

Dry feed samples were ground before analysis.

Sample Extraction. The quantity of a weighed portion depended on the anticipated vitamin B-6 content. The final concentration of the vitamin in the extract was between 0.05 and 2.0 μg/mL. A total portion of 0.1–5.0 g was weighed accurately in a 100-mL calibrated flask, mixed with 25 mL of 5% (w/v) trichloroacetic acid (TCA) and 1 mL of a solution of 4-DOP (100 μg/mL), and then homogenized with an Ultra-Turrax (Wilten Woltil, The Meern, Netherlands). After homogenization, the volume was made up to 50 mL by adding 5% (w/v) TCA. The suspension was vigorously shaken for about 30 min. Samples were transferred into 50-mL screw-capped tubes and centrifuged for 10 min at 2000*g* and 10 °C.

Dephosphorylation. Following centrifugation and filtration through filter paper V 258 (Schut, Heelsum, Netherlands), aliquots of 3 mL of each extract were transferred to 16 mm × 100 mm test tubes and diluted with 0.4 mL of 4 M sodium acetate buffer (pH 6.0). Each step was followed by mixing.

Taka-Diastase (200 mg/mL, 0.1 mL) was added, and the tubes were incubated in a water bath at 45 ± 2 °C for 3 h. Extracts were vortexed for a few seconds every 30 min.

The purpose of the enzymatic treatment was to convert PLP and PMP into PL and PM, respectively. The tubes were cooled to ambient temperaure and 1.5 mL of 16.7% (w/v) TCA was added to precipitate the Taka-Diastase protein. After centrifugation for 10 min at 2000*g* and 10 °C, any cloudy extract was filtered through an 0.45-μm-pore-size membrane (Schleicher and Schuell, Dassel, Germany) prior to HPLC analysis.

Standardization. As vitamin B-6 is light-sensitive, procedures were carried out under subdued light. A working standard solution was obtained by diluting the stock standard solutions of the three free forms and of DOP to concentrations of 0.1–0.2 and 1.0 μg/mL, respectively. An aliquot of 3 mL of the working solution was treated as described above.

Recovery Experiments. For the determination of the recovery, 0.1 mL of each phosphate ester stock solution or 0.1–0.2 mL of the stock solutions of the free forms of vitamin B-6 were added along with 25 mL of TCA prior to blending with the Ultra-Turrax. Specific concentrations of the standards were prepared according to the amount of each vitamer present in each sample. Mixtures were treated as described above.

High-Performance Liquid Chromatography (HPLC). HPLC analysis of vitamin B-6 was carried out by injecting 50 μL of the sample extract onto the ODS Hypersil column using an automatic injector. Unless stated otherwise, the column was eluted with a mobile phase consisting of 3% (v/v) methanol and 1.25 mM 1-octanesulfonic acid (Pic B8) in 0.1 M KH_2PO_4 adjusted to pH 2.15 with H_3PO_4 (flushed with a stream of helium gas for 10 min before use) at a flow rate of 1.2 mL/min. The effluent of the column was mixed with a solution containing 1 M $K_2HPO_4 \cdot 3H_2O$ using a peristaltic pump set at a flow rate of 0.3 mL/min.

The fluorescence of the individual B-6 vitamers was monitored using the fluorescence spectrophotometer set at the excitation/emission wavelength pair of 333/375 nm. The duration of a typical chromatographic run was about 35 min per sample.

Microbiological Assay Procedure. Series of feeds and foodstuffs were analyzed microbiologically for total vitamin B-6 for comparison with the results obtained with the HPLC method. The microbiological assay procedure comprised extraction with sulfuric acid and dephosphorylation of phosphate esters by high-temperature incubation (Kavanagh, 1963; Williams, 1979). *S. uvarum* was used as the test organism and pyridoxine Y medium (Difco Laboratories, Detroit, MI) as the growth medium. The vitamin B-6 concentration of each sample extract was determined relative to a PN standard curve. Turbidity was determined by measuring the absorbance at 660 nm with a Vitatron MCP spectrophotometer (Meyvis, Bergen op Zoom, Netherlands).

RESULTS AND DISCUSSION

HPLC Separation of Pyridoxal, Pyridoxine, and Pyridoxamine. To obtain a good separation of the unphosphorylated B-6 vitamers, the previously described ion-pair HPLC method of Gregory and Sartain (1991) was modified. No gradient but an isocratic mobile phase containing 3% methanol and 1.25 mM octanesulfonic acid in 0.1 M K_2HPO_4 adjusted to pH 2.15 was used by us. For detection, the native fluorescence of the compounds was used. To achieve a more sensitive and selective detection and to avoid observed interferences, e.g. in wheat and rice products, the pH of the mobile phase was elevated by mixing the eluent post column with 1 M K_2HPO_4. Coburn et al. (1983) previously reported on the use of a concentrated phosphate solution for postcolumn pH adjustment. Figure 1 shows typical elution profiles of the working

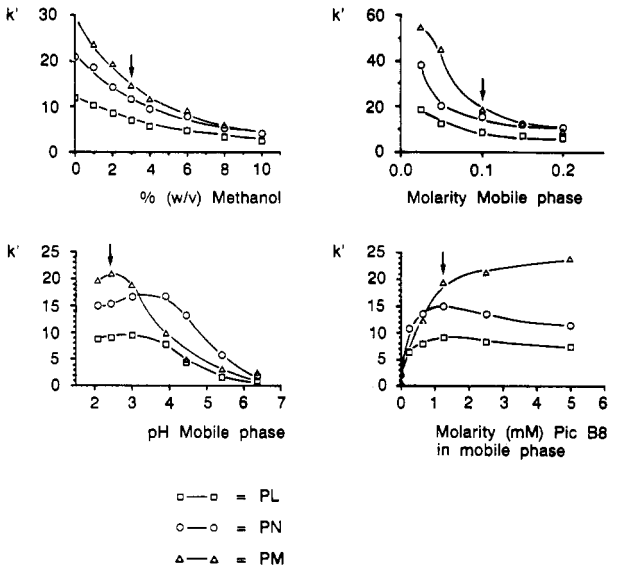

Figure 2. Relationship of the capacity factors (k') of PL, PN, and PM with the different components of the HPLC mobile phase. Unless otherwise indicated, the mobile phase contained 3% (v/v) methanol and 1.25 mM 1-octanesulfonic acid (Pic B8) in 0.1 M KH_2PO_4 adjusted to pH 2.15. The standard composition of the eluent is indicated by the arrows.

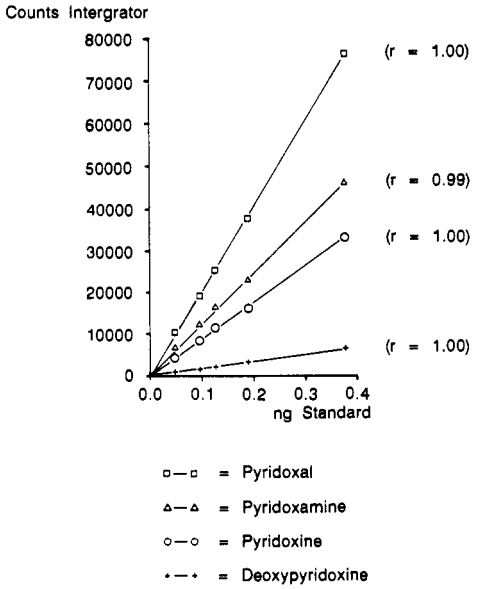

Figure 3. Calibration plot for the various B-6 vitamers.

standard solution and extracts of food samples. The partition of PL, PN, and PM between the stationary and the mobile phase can be influenced by varying the methanol or Pic B8 content or by varying the ionic strength or pH of the mobile phase. Figure 2 shows the relationships between the capacity factors (k') of PL, PN, and PM and the differences in the mobile phase. These results indicate that, if one of the B-6 vitamer peaks of a certain sample is interfered with by sample components, the chromatographic separation may be improved by changing one of the components in the mobile phase.

The compound 4-deoxypyridoxine (DOP), which does not occur naturally in foods and feeds, appeared to be a suitable internal standard on the basis of its stability and complete separation from the other vitamers during HPLC, as also reported previously (Gregory and Fieldstein, 1985) (Figure 1). DOP is structurally closely related to the unphosphorylated vitamin B-6 vitamers, which indicates an extraction behavior comparable to that of these B-6 vitamers.

Table 1. Dephosphorylation (Percent) of PLP and PMP by Enzymatic Hydrolysis after 0–4 h of Incubation

	PLP[a] → PL					PMP[a] → PM				
type of enzyme	0 h	1 h	2 h	3 h	4 h	0 h	1 h	2 h	3 h	4 h
Taka-Diastase	0	76	83	91	93	0	74	95	100	110
Mylase 100	0	46	60	68	77	0	34	41	51	80
acid phosphatase	0	60	77	77	83	0	21	22	39	45

[a] The concentrations of PLP and PMP used were 100 μg/50 mL of extract. Enzyme: 6.66 mg/mL food extract.

Table 2. Recovery of B-6 Vitamers Added to Various Samples Prior to Extraction[a]

	% recovery				
variable	PL	PN	PM	PLP	PMP
n	46	57	47	49	50
mean (%)	100.3	98.4	103.6	95.7	96.6
SD (%)	5.7	6.1	9.6	9.0	8.1
CV (%)	5.7	6.2	9.3	9.4	8.3

[a] The amount of each vitamer added to a sample was comparable to the amount present in the sample.

Table 3. Precision of the HPLC Method in Determining B-6 Vitamers

	within-batch precision (rice)			between-batch precision (corn flour)		
variable	PL	PN	PM	PL	PN	PM
n	4	4	4	4	4	4
mean (μg/g)	0.27	0.76	0.41	0.34	2.17	0.51
SD (μg/g)	0.02	0.02	0.02	0.03	0.08	0.09
CV (%)	7.9	2.5	4.1	9.3	3.8	19.2

Standard solutions were used to construct calibration curves. The response appeared to be linearly related to the injected quantities of the compounds; the correlation coefficients of the straight-line graphs of the three vitamers and the internal standard were better than 0.98 (Figure 3). Assuming the signal-to-noise ratio should be at least 3, the detection limit of the method corresponds to a concentration of 0.02 μg/g for each vitamer. This is sufficient to detect physiologically relevant concentrations in foods and feeds.

Extraction Efficiency. Initial studies were conducted to compare the suitability of several extracting agents. Extraction by H_2SO_4 as described by Bognar (1985) and by HCl, followed by high-temperature hydrolysis, resulted in interferences in the chromatogram (data not shown). Sulfosalicylic acid (SSA) could not be used for all types of food samples (Vanderslice, 1984) and also resulted in interferences during the HPLC procedure described (data not shown). It was found that a TCA extract gave reliable HPLC separation of vitamin B-6 under the conditions employed (Figure 1). To investigate whether the extraction of vitamin B-6 was complete, various weighed portions were analyzed. No significant differences in vitamin B-6 contents were found between 1-, 3-, and 5-g portions of several samples for each of the three free forms. This indicates that the extraction by TCA was complete. Also, the recovery experiments (see below) showed a complete extraction. Previously, it has also been reported that TCA is an effective extractant for vitamin B-6 (Gregory, 1988).

Dephosphorylation Efficiency. Autoclaving in the presence of sulfuric acid (Bognar 1985) in our hands did not result in complete hydrolysis. Extraction with TCA did not result in an appreciable dephosphorylation of PLP or PMP, so that we had to develop a suitable method of hydrolysis. Studies with Taka-Diastase, Mylase 100, and potato acid phosphatase, carried out under different conditions as to incubation time and quantity, were

Table 4. Between-Batch Precision of the HPLC and Microbiological Methods

variable	total amount of vitamin B-6 expressed as PN in corn flour	
	HPLC method	microbiological method
n	4	5
mean (μg/g)	2.93	1.83
SD (μg/g)	0.15	0.28
CV (%)	4.9	15.1

compared. The results in Table 1 show that Taka-Diastase provided quantitative hydrolyses of PLP and PMP into PL and PM, respectively. To investigate whether the enzymatic treatment was applicable to large amounts of the phosphorylated forms, high concentrations of PLP and PMP were added to a food sample (infant formula). The quantity of Taka-Diastase used in this method was capable of hydrolyzing an amount of about 0.5 μg of PLP as well as PMP per milliliter of food extract employed.

The efficiency of dephosphorylation of added PLP and PMP was confirmed by coefficients of variation (n = 50) of 9.4 and 8.3% (Table 2) and average percentages of recovery/conversion of 95.7 and 96.6 for PLP and PMP, respectively.

Precision and Recovery. To determine the within- and between-batch precision and the recovery of both methods of analysis, several equal portions of the same sample (rice for within-batch and corn flour for between-batch comparisons), with and without the addition of PL, PN, and PM, were analyzed in the same series and on different days. The results in Table 3 show that the between-batch coefficient of variation of the HPLC method for the three different forms is very acceptable in

Table 5. Comparison of Results Obtained with the HPLC and Microbiological Methods

type of sample	vitamin content* (μg/g)							
	HPLC method				microbiological method			
						calculated		
	PL	PN	PM	total *a	total b	c	d	e
potato chips	0.50	3.47	1.14	4.95	2.60	47.5		
potato (raw)	0.51	2.34	0.35	3.15	1.95	38.1		
potato (raw, unpeeled)	0.62	2.21	0.38	3.16	1.88	40.5		
potato (cooked)	0.24	2.11	0.62	2.88	1.27	55.9	2.48	48.8
apple (golden rennet)	0.15	0.43	0.04	0.62	0.48	22.6		
bananas	0.71	2.62	2.80	5.71	3.60	37.0		
Brussels sprouts	1.56	2.39	0.42	4.32	2.84	34.3	3.24	12.3
cauliflower	0.63	0.89	0.29	1.77	1.51	14.7		
leek	0.87	2.25	0.32	3.39	2.74	19.2		
sauerkraut	0.17	0.89	0.36	1.36	0.92	32.4		
spinach (frozen)	0.38	0.27	0.37	0.96	0.50	47.9		
kale (frozen)	0.33	0.36	0.38	1.01	0.50	50.5		
pea soup	0.02	0.23	0.28	0.50	0.19	62.0		
grated cheese	0.08	0.10	0.65	0.73	0.40	45.2		
gingerbread	0.35	0.35	0.04	0.74	0.33	55.4		
whole-wheat bread	0.22	1.12	0.37	1.65	1.24	24.8		
whole-wheat macaroni	0.26	2.02	0.47	2.63	2.38	9.5	2.28	-4.4
corn flour	0.34	2.17	0.51	2.93	1.83	37.5		
rice grain	0.38	0.93	0.42	1.73	1.70	1.8		
breakfast porridge	0.35	0.93	0.45	1.66	1.70	-2.4		
hazelnut chocolate	0.84	0.09	0.14	1.05	0.77	26.7	0.83	7.2
chocolate	0.16	0.28	0.08	0.50	0.28	44.0	0.44	36.4
orange lemonade	0.17	0.16	0.23	0.52	0.10	80.8	0.40	75.0
coffee creamer	0.11	0.12	0.04	0.27	0.10	63.0	0.23	56.5
whole butter milk	0.36	0.09	0.09	0.53	0.29	45.3	0.42	31.0
whole milk**	nd**	3.80	nd	3.80	3.70	2.7	3.80	2.6
whole milk powder**	1.40	2.52	0.85	4.64	3.20	31.0	4.01	20.2
canned milk**	nd	4.08	0.10	4.16	3.42	17.8	4.12	17.0
canned milk**	nd	4.38	0.08	4.45	3.50	21.3	4.41	20.6
canned milk**	nd	3.58	0.09	3.66	3.50	4.4	3.62	3.3
canned milk**	nd	3.10	0.09	3.20	3.50	-9.3		
canned milk**	nd	3.50	0.09	3.60	3.80	-5.5		
fruit drink	0.09	0.28	nd	0.37	0.23	37.8	0.35	34.3
orange drink	nd	0.22	0.09	0.30	0.22	26.7	0.26	15.4
smoked beef (horse)	0.58	0.73	6.50	6.84	3.47	49.3	2.85	-21.8
pork collops	4.18	0.44	0.79	5.34	2.05	61.6	2.82	27.3
bacon	0.36	0.45	0.51	1.25	0.82	34.4		
chicken breast	9.42	1.57	1.42	12.28	2.28	81.4	6.00	62.0
guinea pig feed**	3.23	19.59	0.42	23.31	17.2	26.2	22.4	23.4
rabbit feed**	2.64	10.90	0.57	14.04	11.1	20.9	13.3	16.5
rat feed**	2.84	13.62	1.32	17.61	12.2	30.7	16.5	26.1
dog feed**	0.66	3.45	0.23	4.32	3.20	25.6	4.09	21.8
protein concentrate	nd	55.7	nd	55.7	62.0	-11.3		
yeast I	9.90	4.40	12.5	25.0	6.90	72.4		
yeast II	10.9	2.50	17.3	28.3	3.90	86.2		

* a = total amount found in HPLC method, calculated as PN; b = total amount by the microbiological method, calculated as PN; c = $[(a - b)/a] \times 100$ = percent of deviation of microbiological method with respect to HPLC method; d = theoretically calculated content of the microbiological method; e = $[(d - b)/d] \times 100$ = percent of deviation of microbiological method with respect to calculated value. **, fortified with pyridoxine. ***nd, not detectable (below limit of detection).

Table 6. Levels of the Three B-6 Vitamers in Peanut Butter and Frozen Orange Juice Concentrate with and without Enzymatic Treatment with Taka-Diastase, β-Glucosidase, or Both

treatment	peanut butter			orange juice		
	PL	PN	PM	PL	PN	PM
without enzymatic treatment	0.14	0.35	0.21	0.22	1.27	1.91
treatment with β-glucosidase (3 h)	0.20	1.31	0.33	0.35	1.65	1.34
treatment with Taka-Diastase (3 h)	0.27	1.22	0.55	0.24	1.69	2.16
treatment with both enzymes (3 h)	0.21	1.26	0.34	0.36	1.67	1.79
incubation with Taka-Diastase (5 h)	0.18	1.23	0.47	0.22	2.12	2.31
incubation with β-glucosidase (5 h)	0.18	1.26	0.41	0.32	1.93	2.27

[a] Values are given in micrograms per gram; procedure is described in the text.

spite of their low concentrations. The between-batch coefficients of variation for the total amount of vitamin B-6 used in the two methods are given in Table 4. The recovery data for B-6 vitamers added to samples prior to extraction are summarized in Table 2. The precision of the method is confirmed by the average coefficient of variation, which ranged from 5.7 to 9.4% for the recovery of the B-6 vitamers.

Comparison of HPLC and Microbiological Methods. A number of foods and feeds were analyzed for vitamin B-6 by the HPLC method as well as by the microbiological method. Results are given in Table 5. An approximately 40% higher total vitamin B-6 content was found with the HPLC method than with the microbiological method.

In the microbiological assay, the growth responses of *S. uvarum* to the free forms are not equal (100% for pyridoxine, 80% for pyridoxal, and 50% for pyridoxamine) and hardly existent for the phosphorylated forms (19% for pyridoxal 5′-phosphate, 3% for pyridoxamine 5′-phosphate, data not shown). Since the most potent vitamer (pyridoxine) is used in the standard curve, it can be anticipated that the microbiological assay gives an underestimate of the vitamin B-6 content. For the HPLC method, an efficient and complete extraction method and dephosphorylation procedure was developed. It was found that autoclaving in the presence of sulfuric acid, as employed in the microbiological method, resulted in only partial hydrolysis of the phosphate esters (about 80% for PLP and 30% for PMP).

To a limited extent it is possible to calculate the amount of phosphorylated and unphosphorylated vitamers in a certain sample from the differences between HPLC analyses with and without enzymatic treatment. In this way it is possible to estimate the content of phosphate esters. With the knowledge of the differences in growth response of *S. uvarum* (Figure 1), it is possible to calculate the vitamin content expected in the microbiological assay. Pyridoxine 5′-phosphate was not involved in this calculation because of its minor significance as a naturally occurring vitamer (Vanderslice et al., 1980; Coburn and Mahuren, 1983). The formula was as follows: (PL × 0.8 × 1.01) + (PLP × 0.19 × 0.75) + (PM × 0.5 × 0.85) + (PMP × 0.04 × 0.72) + (PN) = theoretical amount of total vitamin B-6 in the microbiological assay. In this approach, no attention is given to the differences in the extraction method of both assays. The use of sulfuric acid has not been validated extensively and may cause a low microbiological value. However, the extraction procedure used in the microbiological assay may result in dephosphorylation. This will result in an overestimate of the value found compared to the calculated vitamin B-6 content in the microbiological assay. It was found that the calculated content was still higher than the content determined with the microbiological assay (Table 5). It should be noted, however, that no significant differences in vitamin B-6 contents were found between the microbiological and the HPLC methods for (semi)synthetic feed or enriched food samples, commonly containing almost exclusively pyridoxine.

An alternative explanation for the difference between the HPLC method and the microbiological method may be found in the presence of glycosylated forms of vitamin B-6. The existence of a conjugated form in various foods of plant origin has been demonstrated in several studies. Kabir et al. (1983a) examined the levels of glycosylated vitamin B-6 in food with a microbiological method using *S. uvarum*. Gregory and Ink (1987) identified pyridoxine 5′-β-D-glucoside as a major form of vitamin B-6 in plant-derived foods. The β-D-glucosides are presumably as stable under our extraction conditions as with the SSA extraction as used by Gregory (1987). However, because Taka-Diastase, the enzyme for dephosphorylation in our study, could have β-glucosidase activity, some experiments have been carried out with orange juice and peanut butter [both reported to be rich in glycosylated B-6 by Kabir et al. (1983)]. Our results showed that incubation with Taka-Diastase or β-glucosidase or both together did not result in significant differences in content of vitamin B-6 in peanut butter, even after 5 h of incubation. Table 6 summarizes the results with and without enzymatic treatment. Apparently, Taka-Diastase may contain some β-glucosidase activity, so that the total amount of vitamin B-6 in plant-derived samples is likely to be overestimated if the bioavailability of vitamin B-6 β-D-glucosides is low (Kabir et al., 1983b; Trumbo et al., 1988). However, for orange juice a small peak between the PL and PN peaks in the chromatogram, tentatively identified as PN 5′-β-D-glucoside, remained after 5 h of incubation in the chromatogram. It should also be noted that the extraction procedure used in the microbiological assay, i.e., autoclaving after the addition of sulfuric acid, probably also leads to the hydrolysis of a large part of the glucosides.

In conclusion, we here report on a vitamin B-6 determination in feeds and foodstuff that is flexible, more reliable, and more sensitive than the classical microbiological assay. Moreover, the described method has some advantages over previously reported HPLC methods, although it is based on the same principles.

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