ng for riboflavin. For some samples, if further clarifications of the extracts are needed, one can use isobutyl alcohol (for thiamin) or chloroform (for riboflavin) to extract the sample filtrate prior to the oxidation step or the UV irradiation step to remove the interfering substances.

With automatic sampler and data processor, the analyst's time can be saved markedly. One operator should be able to analyze at least 20 samples, in duplicate, for thiamin or riboflavin in 1 day provided that samples are prepared and hydrolyzed the day before.

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Received for review October 4, 1979. Accepted January 8, 1980. The mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not mentioned.

Comparison of High-Performance Liquid Chromatographic and *Saccharomyces uvarum* Methods for the Determination of Vitamin B_6 in Fortified Breakfast Cereals

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A previously developed high-performance liquid chromatographic (LC) method was modified to permit its use for the determination of vitamin B_6 in fortified breakfast cereals. The LC method employed an acidic potassium phosphate mobile phase, an octadecylsilica column packing, and detection of the natural fluorescence of the eluted B_6 vitamers. The LC assay of vitamin B_6 in five selected cereals provided consistently high recovery (93.9 ± 7.6%) and precision (coefficient of variability = 4.5 ± 2.7%) and was found to be free of interfering fluorophores. Microbiological assays for total vitamin B_6 using *Saccharomyces uvarum* showed evidence of growth inhibition in four of the five cereal samples. Microbiological assay results based on the mean response for three levels of extract addition to the assay tubes correlated well with the LC results. However, the validity of this comparison must be questioned because of the interference encountered in the microbiological analysis. The LC method was more satisfactory than the microbiological procedure for cereal analysis because of its simplicity, potentially large number of samples which may be analyzed, demonstrated accuracy, and high precision.

Extensive research has been directed toward the development of rapid, sensitive methods for the determination of vitamin B_6 in foods. Because of the limitations of time and precision often associated with conventional microbiological assay methods, recent research has dealt mainly with direct analysis using chemical and physical techniques.

Many complex fluorometric methods have been developed for the determination of vitamin B_6 in foods and biological materials. These have been based mainly on preparative electrophoresis or ion-exchange chromatography, followed by conversion of the B_6 vitamers to either 4-pyridoxic acid or its lactone for fluorometric quantitation (Fujita et al., 1955; Hennessy et al., 1960; Kraut and Imhoff, 1967; Contractor and Shane, 1968; Loo and Badger, 1969; Columbini and McCoy, 1970; Takanashi et al., 1970; Masukawa et al., 1971; Fiedlerova and Davidek, 1974; Chin, 1975; and Gregory and Kirk, 1977). Although the accuracy of these methods has not been extensively examined, the lack of close agreement between fluorometric and microbiological assay results indicates that the fluorometric methods are subject to interference (Kraut and Imhoff, 1967; Chin, 1975; Gregory and Kirk, 1978a,b).

High-performance liquid chromatography (LC) was first applied to the determination of vitamin B_6 in foods by Yasumoto et al. (1975), although application of their procedure is limited by cumbersome gradient elution and post-column derivitization instrumentation. Several simpler LC methods for the separation and ultraviolet absorption detection of the B_6 vitamers have been reported (Williams and Cole, 1975; Wong, 1978; Williams, 1979). However, a lack of chromatographic efficiency and detection sensitivity has precluded the application of these methods to vitamin B_6 assay in foods. Gregory and Kirk (1978b) reported an LC method for the determination of the B_6 vitamers in dehydrated model food systems which was based on an isocratic reverse-phase separation and fluorescence detection. Although this method has been adapted to the determination of 4-pyridoxic acid in urine (Gregory and Kirk, 1979), it has not yet been employed for food analysis. Recently, Vanderslice et al. (1979) reported a method based on anion-exchange LC separation

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0021-8561/80/1428-0486$01.00/0

of vitamin B_6 compounds and fluorometric detection, although this has not yet been applied to food analysis.

The purpose of this study was to examine the applicability of the reverse-phase LC method for the determination of vitamin B_6 in selected vitamin fortified breakfast cereals. The results of the LC assay were compared with those of a conventional microbiological method to assess the relative merits of these procedures for the determination of vitamin B_6.

MATERIALS AND METHODS

Materials. Pyridoxine hydrochloride, pyridoxamine dihydrochloride, and pyridoxal hydrochloride were obtained from Sigma Chemical Co. Pyridoxine Y Medium and *Saccharomyces uvarum* ATCC 1080 were obtained from Difco Laboratories. Distilled water was purified for use in the LC mobile phase by passage through a column of Porapak Q (Waters Associates). All other chemicals were reagent grade.

Six randomly chosen vitamin-fortified breakfast cereals were purchased locally for use as experimental samples. The cereals (approximately 30 g each) were finely ground in a Waring blender and stored at −10 °C in sealed glass containers until analyzed by microbiological and LC methods for vitamin B_6.

LC Assay Procedure. The cereal samples were assayed for vitamin B_6 using a slight modification of the reverse-phase LC method previously described (Gregory and Kirk, 1978b). This procedure is based on the use of a 0.033 M potassium phosphate, pH 2.2, mobile phase, and a μBondapak C_{18} column (0.4 × 30 cm; Waters Associates).

Each cereal was analyzed in triplicate with triplicate parallel recovery samples. Two-gram portions of each cereal were accurately weighed, placed in individual 25-mL volumetric flasks, and suspended in 15 mL of 0.5 M potassium acetate, pH 4.5. For recovery samples, 1 mL of a solution containing 30 μg each of pyridoxamine, pyridoxal, and pyridoxine was added at this point. The flasks were sonicated for 30 min at ambient temperature in a 125-W ultrasonic bath (Fisher Scientific Co.).

After sonic treatment, the contents of the flasks were centrifuged at ambient temperature at 4000*g* for 20 min to sediment insoluble components. Preliminary trials indicated that the content of vitamin B_6 5′-phosphate esters was negligible in fortified cereals. Thus acid phosphatase treatment was omitted. Ten-milliliter aliquants of each 4000*g* supernatant were mixed with 2.5 mL of 33.3% (w/v) trichloroacetic acid (Cl_3CCOOH) in polypropylene centrifuge tubes, held in a 50 °C water bath for 15 min, and centrifuged at 10000*g* for 20 min at ambient temperature. The supernatant solutions were filtered through a 0.45-μm pore size membrane prior to chromatographic analysis.

LC standards were formulated from a 10 μg/mL stock mixture of pyridoxal (PL), pyridoxine (PN), and pyridoxamine (PM), which was prepared by dissolving 12.2 mg of PL-HCl, 12.2 mg of PN-HCl, and 14.2 mg of PM-2HCl in 1 L of 0.5 M potassium acetate, pH 4.5. Working standards were prepared by transferring 0.625–6.25 mL of the stock solution to 25-mL volumetric flasks containing 5 mL of 33.3% (w/v) Cl_3CCOOH and diluting to 25 mL with the pH 4.5 acetate buffer. This provided working standards over a range of 0.25 to 2.5 μg/mL for each free base B_6 vitamer.

Chromatographic analysis was performed at a flow rate of 2.0 mL/min, providing an operating pressure of about 1300 psig. Fifty-microliter volumes of samples and standards were injected using a filled loop technique. The B_6 vitamer content of the cereal extracts was calculated using interpolation from the respective standard curves based on peak height, employing a correction for the recovery of the internal standards.

The analyses were performed using an Altex Model 312 liquid chromatograph and an American Instrument Co. FluoroMonitor detector. The FluoroMonitor was equipped with a 70-μL flow cell, a Germicidal lamp (General Electric, Model C4T41), a 295-nm interference excitation filter (American Instrument Co.), and a 405-nm narrow pass emission filter (Turner Associates).

Microbiological Assay Procedure. Total vitamin B_6 in the cereal samples was determined using the basic method of Haskell and Snell (1970). This procedure was found in preliminary trials to provide equal molar response for PL, PM, and PN. Each cereal sample (0.25 g) was extracted in duplicate in 180 mL of 0.44 N HCl at 121 °C for 2 h, followed by adjustment to pH 4.5 with 3 N KOH and dilution to 250 mL. After filtration, the extracts were assayed using 0.05-, 0.10-, and 0.15-mL levels of addition to the assay tubes.

A parallel recovery sample was prepared by adding 3.75 μg of PN prior to the extraction. Recovery values ranged from 85 to 100% for the internal PN standard. A standard curve was run over the range of 0 to 8.0 ng of PN per assay tube in each analysis. Triplicate assay tubes were run for sample extracts and standards at each level of addition. The growth period was 18 h at 29 °C in a water bath shaking at 120 oscillations/min. Turbidity was measured at 650 nm using either a Bausch and Lomb UV200 or Gilford Model 250 spectrophotometer.

The content of apparent vitamin B_6 in each assay tube was estimated from plotted standard curves. These data were employed to calculate the apparent cereal vitamin B_6 concentration. For each cereal, the response of all 18 assay tubes was employed in the estimation of the mean vitamin B_6 concentration. Also, standard deviations were calculated on the basis of all 18 data values.

Because of the photolability of vitamin B_6 compounds, all LC and microbiological assays were performed in the absence of incandescent and white fluorescent light. When necessary, lighting was provided with gold fluorescent lamps (General Electric, Model F40G0) to minimize photochemical degradation of the B_6 vitamers.

Statistical Analysis. The results of vitamin B_6 determinations using microbiological (mean) and LC methods were compared statistically using one-way analysis of variance. The significance of the slope of linear regression plots was determined using a two-tailed *t*-test. All procedures were described by Neter and Wasserman (1974).

RESULTS AND DISCUSSION

The experimental samples used in this study were arbitrarily selected. The cereals analyzed provided products with a broad range of gross composition, color and flavor additives, and processing methods upon which to compare the LC and microbiological assay procedures.

The LC assay method was found to be a sensitive, simple procedure for the determination of vitamin B_6 in the cereal products. A typical chromatogram is presented in Figure 1, illustrating the good resolution and satisfactory chromatographic efficiency (approximately 1700 theoretical plates for PN). Under these operating conditions, the retention times for PM and PL were approximately 3.0 and 4.3 min, respectively. These vitamers were not detected in the cereal samples at the present detection limit of 0.1–0.5 μg/g. As expected, PN was the sole detectable form of vitamin B_6 because of its natural occurrence in cereal grains and its addition in fortification as PN-HCl. Chromatograms with simplicity similar to that of Figure 1 were obtained with all cereals examined in this study.

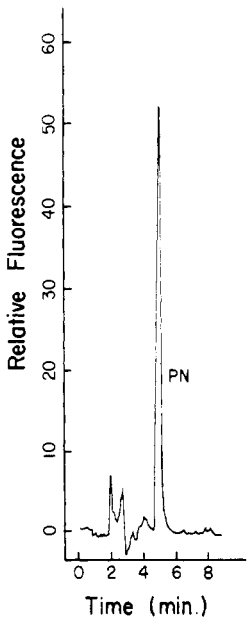

Figure 1. Chromatogram from LC determination of vitamin B_6 in Fruity Pebbles. Conditions are described in the text.

Table I. Recovery of Pyridoxine (1.2 μg/mL) Added before Extraction of Cereals in LC Analysis

cereal	% recov[a]
Post Toasties	76.3 ± 9.2
Super Sugar Crisp	104.0 ± 5.1
Cocoa Pebbles	99.3 ± 8.7
Fruity Pebbles	96.5 ± 7.8
Rice Krispies	93.6 ± 7.0

[a] Percentage recovery values represent mean and standard deviation of triplicate determinations.

Fluorescence spectral examination (excitation and emission) of collected PN LC peaks confirmed the absence of interfering compounds.

The quantitative results of the LC assay are presented in Tables I and III. The percentage recovery of PN added prior to extraction was found to vary between samples (Table I), although the recovery was high in all cases. The precision of the recovery data was satisfactory, as indicated by a coefficient of variability of 7.2 ± 3.5% (CV ± standard deviation). The vitamin B_6 (PN) content data for the cereals (Table III) exhibited high precision (coefficient of variability 4.5 ± 2.7%). These results, along with the spectrally demonstrated absence of interference, strongly support the validity of the LC assay method for vitamin B_6 in fortified cereal products.

The *S. uvarum* assay for total vitamin B_6 was performed to provide a comparison of the results of this conventional method and the LC procedure. The microbiological results provided evidence of interference, however, making the numerical validity of this comparison somewhat uncertain. The apparent vitamin B_6 concentration (micrograms/gram) of the cereals, as determined microbiologically, is shown in Table II as a function of extract volume per assay tube. Four of the five cereals exhibited a significant inverse relationship between apparent vitamin B_6 (micrograms/gram) and the volume of extract added. Only the Rice Krispies did not exhibit this "negative drift" phenomenon. In an effort to compensate for the response inhibition, linear regression equations were calculated and the *Y*-intercept values (i.e., μg/g predicted for 0.00 mL of extract/assay tube) used as estimators of vitamin B_6 in the cereals (Table II).

Table III provides a summation of the results of the microbiological assay, calculated either as the mean or the extrapolated *Y*-intercept response, and the LC results. The vitamin B_6 content calculated by the *Y*-intercept method for microbiological data evaluation was greater than the LC results for each cereal which exhibited a significant "negative drift". In contrast, when the microbiological results were calculated as the mean of the observed responses for each cereal, good correlation was found with the LC data. Because the calculation based on the mean microbiological response neglects the apparent bias of the dose-dependent yeast growth inhibition, this high correlation with the LC results may be fortuitous. In addition, the microbiological results based on the *Y*-intercept extrapolation method are subject to a high degree of theoretical uncertainty because the linearity of the microbial response at low levels of extract addition (<0.05 mL) was not determined.

Although similar microbiological methods for total vitamin B_6 are commonly used in food analysis, biases of this "negative drift" type have not been widely reported. These represent a potential source of error in all microbiological assays (Pearson, 1967) and may profoundly affect the accuracy of the results. Recently, Voigt et al. (1979a,b) have evaluated several sources of error in vitamin analysis by microbiological methods. These authors reported that the KCl formed during extract neutralization may inhibit *S. uvarum* growth when present in high concentrations in the assay tubes (>620 mM). As the volumes of addition employed in the present study were small (0.05–0.15 mL), any effect of the added KCl (≤13 mM) would be negligible. Voigt et al. (1979a) also reported that certain antimicrobial preservatives may interfere with microbiological assays. The low water activity of the cereals obviates the need for such preservatives and thus rules out this potential source of error. Other sources of interference in the *S. uvarum* method for total vitamin B_6 have not been identified. The effectiveness of preparative ion-exchange chromatography for the removal of interfering compounds should be examined. Also, the susceptibility of other assay organisms

Table II. Vitamin B_6 Concentration in Cereals Determined by *S. uvarum* Assay

	mL of extract added/tube[a]			regression parameters[b]			
cereal	0.05	0.10	0.15	b_0	b_1	r	P
Post Toasties	48.8	27.1	34.4	48.6	−125.8	−0.479	0.06
Super Sugar Crisp	50.2	43.2	40.5	52.9	−93.3	−0.581	0.01
Fruity Pebbles	80.0	58.5	47.4	92.8	−303.0	−0.968	0.01
Cocoa Pebbles	61.5	53.4	46.6	70.7	−178.3	−0.932	0.01
Rice Krispies	31.2	36.0	34.5	31.6	5.6	−0.035	NS

[a] Each value is the mean observed for the duplicate extracted samples of each product. Data represent micrograms of vitamin B_6/gram of cereal. [b] Linear regression parameters: b_0 = *Y* intercept (micrograms of vitamin B_6/gram of cereal); b_1 = slope (μg g^{-1} mL^{-1}), r = correlation coefficient; P = level of significance of slope (NS = not significant). The linear regression parameters are shown for milliliters of extract/tube (x) vs. apparent micrograms/gram (y).

Table III. Summary of Assay Results for Vitamin B_6 in Breakfast Cereal Samples as Determined by Microbiological and LC Methods[a]

	microbiological calculation employed[b]		
cereal	Y intercept	mean	LC[c]
Post Toasties	48.6	35.6 ± 10.9	44.2 ± 0.8
Super Sugar Crisp	52.9	43.5 ± 6.8	41.8 ± 1.0
Fruity Pebbles	92.8	62.5 ± 13.1	64.9 ± 4.9
Cocoa Pebbles	70.7	52.8 ± 8.1	52.7 ± 1.4
Rice Krispies	31.6	32.1 ± 6.6	35.5 ± 1.6

[a] Data represent micrograms of vitamin B_6/gram of cereal. Mean ± standard deviation. [b] Methods used in microbiological assay quantification: "Y intercept" = Y intercept of linear regression curve of micrograms/gram (x) vs. milliliters of extract/tube (Y); "mean" = conventional method based on the mean of the observed responses. [c] LC = high-performance liquid chromatography.

such as *Tetrahymena pyriformis* and *Kloekera brevis* should be investigated.

The results of this study indicate that the reverse-phase LC procedure provides a rapid, precise, and accurate technique for the determination of vitamin B_6 in fortified cereals. The problems encountered in the direct microbiological assay for total vitamin B_6 in these products, in addition to the cumbersome nature and poor precision of the method, represent serious analytical limitations. Research is presently in progress concerning the application of this basic HPLC procedure to the determination of the naturally occurring B_6 vitamers in more complex food matrices.

ACKNOWLEDGMENT

The technical assistance of Doris Manley and the statistical consultation of John Cornell, University of Florida, Statistics Department, are gratefully acknowledged.

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Received for review August 28, 1979. Accepted December 13, 1979. Florida Agricultural Experiment Station Journal Series No. 1909. This research was supported by funds from the Florida Agricultural Experiment Station.

Automated Procedure for Routine Analysis of Tryptophan in Cereal and Legume Food Samples

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Tryptophan is one of the most important amino acids. Quantitative determination of tryptophan is still difficult. The various methods of quantitative analysis published so far and currently used are not well adapted to routine determination of tryptophan in vegetable food samples. The method described here is based on a colorimetry principle used for tryptophan analysis in pure proteins. The ground flour samples (40 μm) of cereal and leguminous plants are hydrolyzed with barium hydroxide and are analyzed by a two-step automatic colorimetry process with *p*-dimethylaminobenzaldehyde and sodium nitrite. Interferences from the various seed components and especially from pigments absorbing at 590 nm have been eliminated. The hydrolysis conditions have been optimized in order to allow routine analysis of samples for our breeding programs. The procedure permits the analysis of more than 50 samples/day and per person. The whole process has a reproducibility of 4%. The sensitivity of this method enables quantification of very small amounts of tryptophan (less than 10 μg/mL) with accuracy.

The knowledge of the essential amino acid composition and especially the amount of tryptophan is necessary to determine the protein quality of leguminous or cereal seeds (Mitchell and Block, 1946; Oser, 1964). Following the procedure of Moore and Stein (1951), acid hydrolysis with HCl 6 N is used to release most of the amino acids which can then be quantified by ion-exchange liquid chromatography. Under such conditions, tryptophan is destroyed and cannot be directly determined in acid hydrolysates just

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0021-8561/80/1428-0489$01.00/0