COMPARISON OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHIC, GAS LIQUID CHROMATOGRAPHIC, AND SACCHAROMYCES UVARUM METHODS FOR THE DETERMINATION OF B_6 COMPOUNDS

by

Kim L. Lim

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APPROVED:

J. A. Driskell, Chairman

J. K. Palmer

J. A. Phillips

R. W. Young

S. J. Ritchey

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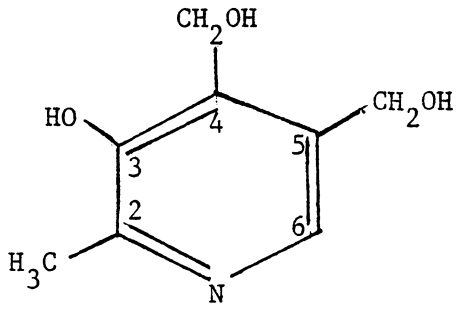
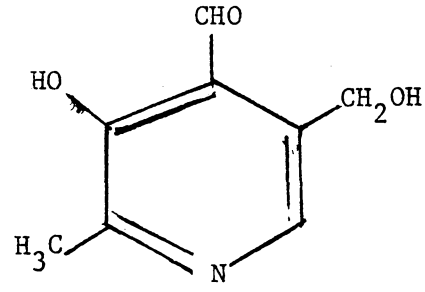
INTRODUCTION

Vitamin B_6 occurs in natural foods in various forms, mainly as pyridoxol (pyridoxine, POL), pyridoxal (PAL), pyridoxamine (PAM), pyridoxamine-5-phosphate (PAMP), and pyridoxal-5-phosphate (PALP) [structural formulas are shown in Figure 1]. Quantitative analyses of vitamin B_6 have been achieved by microbiological methods (1-14). There are, however, several disadvantages in the use of these methods: 1) these procedures can be lengthy, often requiring 20 hr or more of analysis time, 2) total vitamin B_6 levels are measured only without regard to the vitamers present, 3) there is variability in growth response to vitamin B_6 with various microorganisms, and furthermore 4) microbiologically unavailable complexes of the vitamin may be formed as a result of acid extraction or microbial growth may be retarded by substances formed in the food extract thus affecting the accuracy of an assay (15).

There has also been considerable work done toward the development of chemical methods in the determination of vitamin B_6 in foods. Fluorometric methods are often used (16-19), although colorimetric and spectrophotometric techniques are also employed. These physico-chemical methods are all subject to the problem of interfering impurities which can seriously affect the accuracy of the measurements.



PYRIDOXINE
(POL)
MW 169.184



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(PAL)
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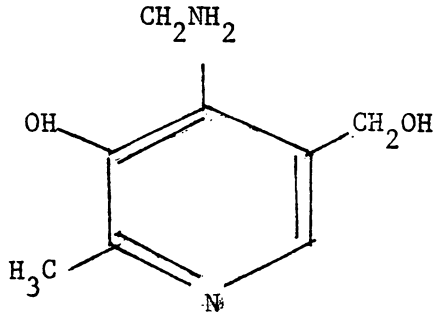
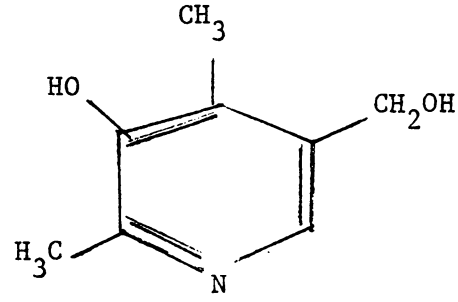


PYRIDOXAMINE
(PAM)
MW 168.200



DEOXYPYRIDOXINE
(DPOL)
MW 153.175

Fig. 1 -- Pyridoxine and related compounds

Gas chromatography (GC) is one of the most important and widely used laboratory separation techniques to date (20). Recent advances in the techniques of GC offers a potential method for the detection and quantitative determination of the various forms of vitamin B_6 in foods and similar materials with satisfactory specificity, sensitivity, convenience, and reduction in analysis time. To date, studies for the quantitation of vitamin B_6 using gas chromatography have been conducted using only pure reagent standards of the various vitamin B_6 compounds, primarily POL, PAL, and PAM. Recently interest has been revived in the development of theory and the instrumentation of liquid chromatography that has made possible faster and more efficient analyses (20). This development followed and was catalyzed by the development of GC (21). There are many similarities between liquid chromatography and GC, the two techniques in theory are very similar and in practice they are complementary.

Recent developments in hardware and packings for high performance liquid chromatography, which is synonymous with high pressure liquid chromatography (HPLC), have overcome the problems of non-reproducible behavior and low efficiency separations previously associated with column chromatography making it competitive with GC in speed and resolution of complex mixtures (22). HPLC has been applied to the quantitative analysis of analgesics (23), pesticides (24, 25), and fat-soluble vitamins (26, 27) with a precision and accuracy of retention time and peak area measurements of about 1.0%. Separation of some water-soluble vitamins by HPLC have been reported by various researchers

(15, 26, 28, 29, 30, 31-33, 34) using columns utilizing ion-exchange (15, 29, 30, 34), reverse-phase (31, 32), bonded-phase (17), or liquid-solid (28, 33) chromatography.

With the introduction of a new and better reagent for vitamin B_6 derivatization N-methyl-bis-trifluoroacetamide (35) and the enhancement in sensitivity with the use of an electron capture (EC) detector (35, 36), it was proposed in this study to explore the possible use of a GC system equipped with a ^{63}Ni EC detector for the separation and quantitation of N-methyl-bis-trifluoroacetamide derivatives of B_6 vitamers in selected foods. Results of the quantitative analyses on some selected foods were compared with results obtained by microbiologic and HPLC methods. HPLC is a complementary separation technique which is fast becoming competitive with GC.

A major advantage associated with the use of HPLC is that a minimum of sample clean-up is required. Even if clean-up is necessary, derivatization (as in GC) is not needed for analysis of vitamin B_6. In contrast to GC the recovery of the B_6 compounds in the original form and quantity is also possible. An attempt was also made in this study to develop an HPLC method (using a bonded-phase column) for the separation and quantitation of vitamin B_6 compounds in selected food materials. The results of the HPLC assay method were compared to data obtained from GC-EC and microbiologic procedures.

REVIEW OF THE LITERATURE

Korytnyk et al. (37) and Korytnyk (38) investigated the gas chromatographic properties of isopropylidene, acetyl,3-O-benzyl, and trimethylsilyl derivatives of several compounds of the vitamin B_6 group. These derivatives were found amenable to determination by flame ionization after separation on a column of silicone gum rubber; peak areas of pyridoxal acetate showed a linear increase in the range of 0.8 to 15.0 g. Trimethylsilyl derivatives are particularly useful for the gas chromatographic separation for the phosphate forms of the vitamin and some of its analogues (37). Satisfactory separation of the acetyl derivatives of PAL, POL, and PAM with subsequent detection by flame ionization and beta-ionization detectors was reported by Prosser and Sheppard (39).

Several investigators (40-42) studied and utilized the trimethylsilyl derivatives of vitamin B_6 for analyses of the various vitamers. These authors reported good separation and detection of the vitamin derivatives in addition to simple and reproducible derivatization.

Imanari and Tamura (43) examined the gas chromatographic separation of the trifluoroacetyl derivatives of POL, PAM, PAL-methyloxime, and pyridoxic acid lactone. These compounds exhibited a high electronegative nature and were completely separated. Detection by EC techniques showed

a good response to 0.1 ng of POL derivatives, 2.0 ng of PAL derivatives, and 0.2 ng of PAM derivatives.

Sennello and Argoudelis (44) described the chromatographic determination of pyridoxine in a multivitamin preparation using N,O-bis-(trimethylsilyl)-acetamide as a derivatizing reagent. A linear response of 2.0 to 10.0 g was obtained with the use of a thermal conductivity detector.

Sheppard et al. (45) and Prosser et al. (46) described methods for the determination of vitamin B_6 in pharmaceutical products involving acetylation of the vitamin followed by analysis with GC. Results were confirmed by use of the following analytical techniques: infrared, ultraviolet, nuclear magnetic resonance, and mass spectrometry (46). A similar method utilizing an acetyl derivative of vitamin B_6 was described by Korytnyk (47) who reported that the fully acetylated derivatives of vitamin B_6 were suitable for fast and quantitative separation of the 3 simple forms of the vitamin (but not the phosphates) and the most important metabolite pyridoxic acid with the use of GC. Separation of the 3 vitamers PAL, POL, and PAM and 4-pyridoxic acid lactone was accomplished within 13 min with the use of programmed temperature GC.

Williams (36) separated the heptafluorobutyryl derivatives (derivatization reagent, heptafluorobutyrylimidazole) of PAL, POL, and PAM by means of GC. Data presented suggested that quantitative determinations of POL were possible at least within the range of 1.0 to 10.0 ng and for PAL and PAM, within the range of 2.0 to 20.0 ng using a EC

detector. Patzer and Hilker (35) reported the use of a new reagent, N-methyl-bis-trifluoroacetamide for vitamin B_6 derivative formation which offers the advantage of a rapid, clean, and simple analytical procedure. The hydrochloride of PAL, POL, and PAM were used and a detection minimum of at least 250 ng was found using a flame ionization detector.

Wildanger (48) in a comprehensive review discussed the various tools of HPLC. Analyses are shown which are interesting for food chemistry (including vitamin analyses) and future possibilities were discussed. Van de Weerdhof et al. (28) illustrated the usefulness of liquid chromatography with the routine analyses of vitamin A, beta-carotene, thiamin, and riboflavin in foods. The authors reported that it was possible to carry out 25 vitamin A and beta-carotene analyses or 25 thiamin and riboflavin analyses per day and obtain a standard deviation of about 3.0%. Conrad (31, 49) reported on the application of HPLC to the rapid analyses of food nutrients, and included a discussion of the apparatus and working parameters with respect to the analyses of some selected fat- and water-soluble vitamins in foods (cereals, feed additives, infant formula, plant foods, syrups, commercial honey, and juices).

Talley (50) described an HPLC method for the separation of several monosubstituted pyridine isomers (isomeric mixtures of the cyano-, carboxamido-, and carboxy-substituted pyridines) which yielded quantitative results with a relative standard deviation of about 1.0%.

Separation and quantitative analyses of the water-soluble vitamins: nicotinic acid, thiamin, riboflavin, ascorbic acid, folic acid, POL, PAL, and PAM were carried out by Williams et al. (15) with the use of high speed ion-exchange chromatography with superficially porous ion-exchange column packings. The reproducibility of retention times and peak areas were shown to be better than 1.5% and the lower limits of sensitivity for the test vitamins were better than 50.0 ng. The method afforded a fast analysis time and minimum of sample clean-up. These methods have been applied to vitamin analysis of pharmaceutical products and selected foods.

Williams and Cole (16) reported the separation of PAL, POL, and PAM by means of high pressure ion-exchange chromatography using both single buffer and gradient elution techniques. Ultraviolet light absorption provided a sensitive and quantitative means of detection. Gradient elution was found to considerably reduce interference due to contaminating materials. POL was successfully analyzed quantitatively in commercial vitamin capsules. Attempts to analyze foodstuffs such as chicken muscle, dried beans, and whole wheat were not successful due to many overlapping peaks which made quantitation and identification of the vitamins difficult. It is possible that a clean-up of these natural materials may have rendered the method more effective.

Wills et al. (17) reported the resolution of water-soluble vitamins including vitamin B_6 by HPLC on two bonded-phase columns, μBondapak-C_{18} and μBondapak-NH_2. The differences in separation and retention times of individual vitamins of multivitamin samples were determined using varying

proportions of water:methanol as the the eluting solvent and by addition of various salts, buffer solutions, and paired-ion-chromatography reagents to the water:methanol. MicroBondapak-NH_2 was reported as being more suitable than μBondapak-C_{18} for the analyses of POL. Application of this method to foods will require an individual examination of each type of food for the presence of interfering substances and the development of techniques for their removal. Callmer and Davies (34) have also reported a rapid separation and quantitative determination of the water-soluble vitamins B_1, B_2, B_6, and nicotinamide in multivitamin preparations using HPLC on a pellicular cation-exchange resin.

Gregory and Kirk (51) had used a reverse phase HPLC technique which included a μBondapak C_{18} column, 0.033M potassium phosphate, pH 2.2 mobile phase, and fluorescence detector to assay for vitamin B_6 in a study of storage effects on vitamin B_6 stability and bioavailability in dehydrated food systems fortified with B_6 vitamers. Utilizing a similar reverse phase HPLC technique, Gregory (52) compared the results of microbiological and HPLC methods for total vitamin B_6 with rat bioassay data for biologically available vitamin B_6 in nonfat dry milk and a fortified rice breakfast cereal product. This reverse phase HPLC technique was also used by Gregory (53) to compare the HPLC and Saccharomyces uvarum (ATCC No. 9080) methods for the determination of vitamin B_6 in fortified breakfast cereals.

Stewart and coworkers (54) reported on the qualitative and quantitative analysis of an isoniazid-pyridoxine hydrochloride mixture by HPLC using an octa-decyl column and absolute methanol:water (60:40)

(pH 2.5) containing 0.01M dioctyl sodium sulfosuccinate. Chromatographic separation of the drug mixture was accomplished using ion pair formation.

O'Reilly and coworkers (55) described an HPLC assay method for the rapid and specific determination of pyridoxine and some of its metabolites, pyridoxal and 4-pyridoxic acid, in human plasma and urine after oral administration of the pyridoxine hydrochloride at psychiatric dose levels. Separation was carried out on a Partisil 10-ODS particle size 10 mm column with UV detection at 291 nm. The solvent used was 0.067 M potassium dihydrogen phosphate solution in double-distilled water, to which 10 ml of a phosphoric acid solution (40%) were added per L (final pH, 2.6).

Vanderslice et al. (56) developed a HPLC system for the separation, identification, and determination of POLP, PAMP, PALP, PAL, PAM and POL. The system included a Bio-Rad A-25 resin column, NaCl:glycine:NaOH and NaCl:glycine:HCL buffers with detection by fluorometry. Using a similar HPLC method Vanderslice and coworkers (57, 58) have developed a procedure to identify and quantify B_6 vitamers in various foods. The method uses sulfosalicylic acid as an extraction agent with subsequent qualitative and quantitative analysis by anion exchange chromatograpy. Vanderslice et al. (59, 60) have also separated and quantified B_6 vitamers in plasma using a slightly modified HPLC system as described above.

The classical method of assay for vitamin B_6 in foods using Saccharomyces uvarum has several disadvantages such as lengthiness of procedure and variability in growth response of the test organism. In

contrast, recent developments in HPLC technology now permit a simple and rapid analytical procedure for many different compounds including water-soluble vitamins. This study was designed to investigate the use of HPLC and GC-EC methods for the qualitative and quantitative analyses of B_6 vitamers in selected foods as an alternative method to the classical analytical method using Saccharomyces uvarum.

PROCEDURES

This study was designed to investigate the use of HPLC and GC-EC for the qualitative and quantitative analyses of B_6 vitamers in selected foods as alternative methods and to compare data obtained by these methods to that obtained by the classical analytical method using Saccharomyces uvarum (ATCC No. 9080).

Selection of foods:

A. Foods

Three diverse food materials (bread, milk, and peas) representative of the cereal, dairy, and vegetable food groups respectively were selected for analysis of B_6 vitamer content.

B. Sources

Brand names of bread, milk, and peas were purchased from two local grocery stores: Kroger Company (K) and Radford Brothers, Incorporated (R). Description, sample sizes and number of units of samples purchased for analyses are shown in Table 1.

Sample preparation

A. Precautions

The B_6 vitamers are photosensitive; thus precautions were taken throughout this study to protect them from light (especially UV light)

Table 1 -- Foods used for the analysis of contents of B_6 vitamers

Food	Brand	Description and sample size	Vendor	No. of units of sample
Bread	Rainbo	Enriched white bread	K*	3 loaves
	Brand	Net wt. 567g	R**	3 loaves
Milk	Carnation	Instant non-fat dry milk.	K	2 boxes
		Fortified with vitamins A&D.	R	2 boxes
		Pasteurized - extra grade.		
		Net wt. 716.8g		
Peas	Green Giant	Very young tender sweet peas.	K	3 cans
		Net wt. 482g	R	3 cans
		Wt. of peas 288.4g		

* Kroger Company, Blacksburg, Virginia store.

** Radford Brothers, Inc., Blacksburg, Virginia.

during treatment and storage. Precautions included--drawn shades at the windows, use of photographic red light, wrapping the foods with aluminum foil when necessary, and placed in amber colored glass vessels for storage. All foods were stored at 5°C when appropriate.

B. Treatment and analysis design

Duplicate samples from each vendor were prepared for subsequent analyses with HPLC, GC-EC, and microbiological assay. Two injections were made for the HPLC from each prepared sample and similarly two injections were made for the GC-EC from each prepared sample (Fig. 2).

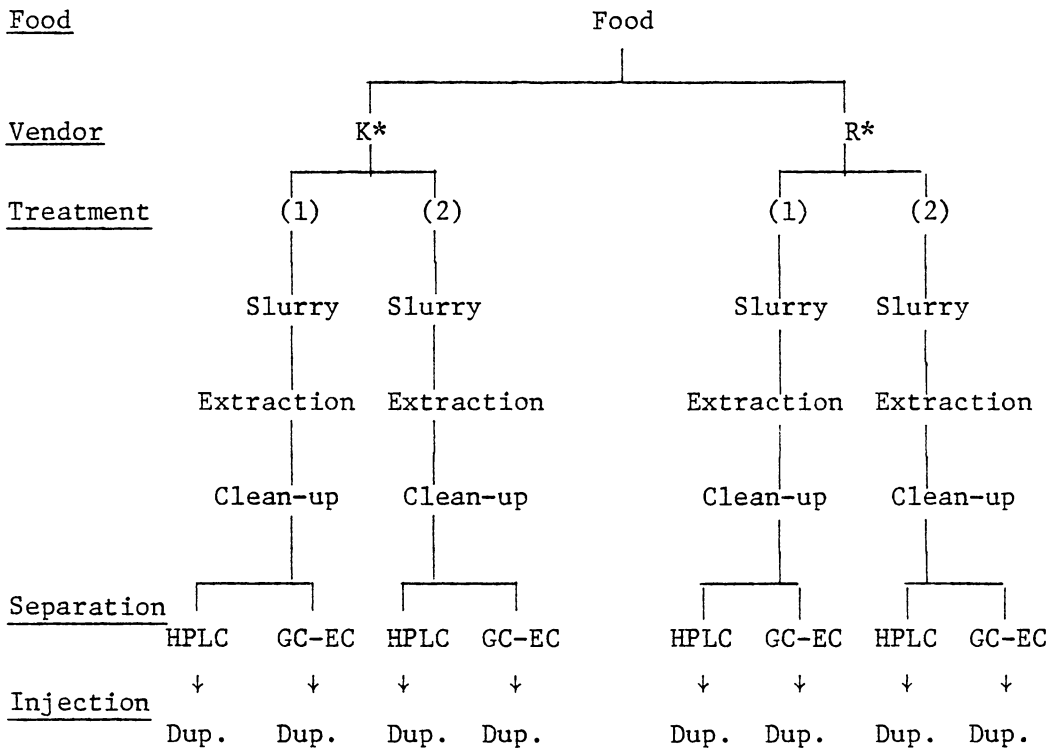
C. Homogenization of food samples

Three different methods were used to obtain a homogeneous mixture for extraction and subsequent treatments; each method being appropriate to the physical characteristics of the particular food.

1. Bread

The three loaves of bread (Fig. 3) were shredded manually into a large mixing bowl and then passed twice through a number 1551 Climax food chopper (Universal Chopper Div., Union Manufacturing Co., Meriden, Connecticut).

Two hundred grams double-distilled water was added to a 100 g portion of the ground bread. The mixture was homogenized for 5 min at about 20,000 rpm in a Model 700 single speed Waring blender (Waring Prod. Corp., New York, NY). The slurry was then transferred into a 500 ml beaker, covered with a plastic wrap and aluminum foil, and stored in a refrigerator at 5°C until used (24-48 hr).



* Kroger Company, Blacksburg, Virginia store.

** Radford Brothers Co. Inc., Blacksburg, Virginia.

Figure 2 -- Treatment and analysis design

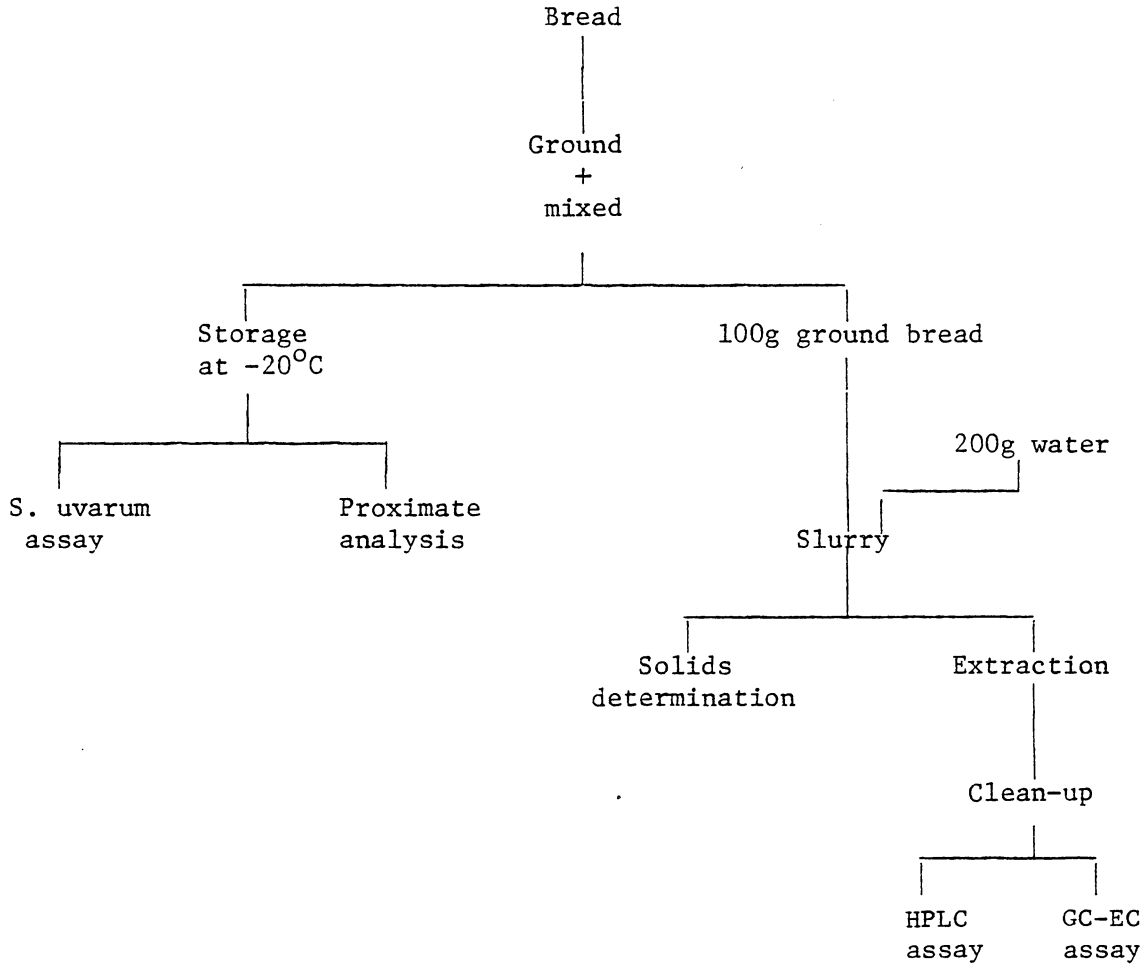


Figure 3 -- Preparation of bread sample

2. Milk

The contents from the 2 boxes of Instant non-fat dry milk (NFDM) were passed twice through a number 20 mesh-size sieve (Fig. 4) into a 4 L mixing bowl and then covered with aluminum foil. A 100 g portion of the milk powder was then reconstituted with 200 g double-distilled water to form a slurry in a 500 ml beaker with mechanical stirring. The slurry was then covered with plastic wrap and aluminum foil and then stored in a refrigerator (5°C) until used (24-48 hr).

3. Peas

The contents of 3 cans of peas were drained through a large commercial sieve for 10 min and the juice collected (Fig. 5) in a 4 L mixing bowl.

The weight of the drained peas was determined. An equal weight of liquid (juice + double-distilled water as needed) was added to the peas. The mixture was blended for 5 min at medium speed (about 14,000 rpm), 1 min at high speed (about 17,000 rpm) and then for another 1 min at medium speed in a Model CB-4 Waring blender (Waring Product Corp., New York, NY) to yield a slurry. The slurry was then contained in a 500 ml beaker covered with plastic wrap and aluminum foil and stored in a refrigerator at 5°C until needed (24-48 hr).

4. Storage of food samples for S. uvarum assay and proximate analyses.

All remaining portions of the 3 foods after the slurries were made were frozen at -20°C for future S. uvarum assay and proximate analyses. Ground bread and milk powder were each stored in labeled Dow Ziploc storage seal bags (DOW Chemical Co., Indianapolis, IN.) and the bags

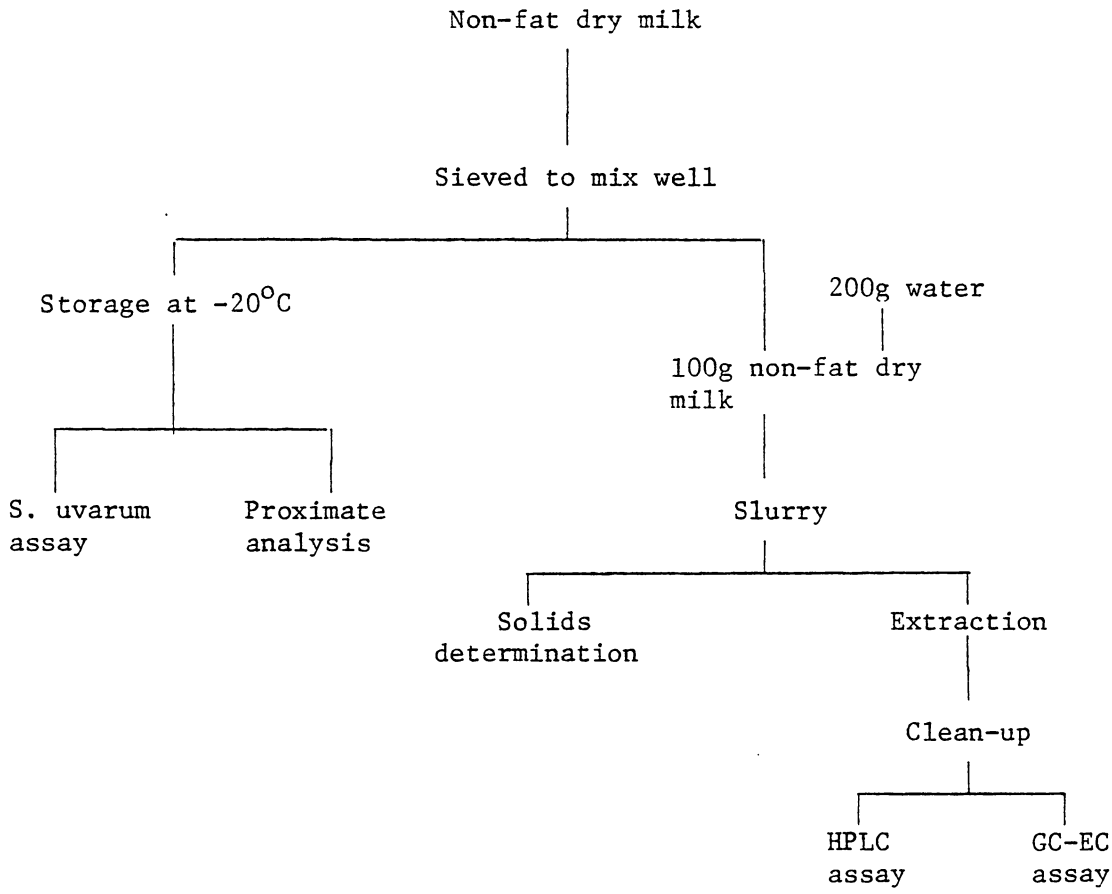


Figure 4 -- Preparation of milk sample

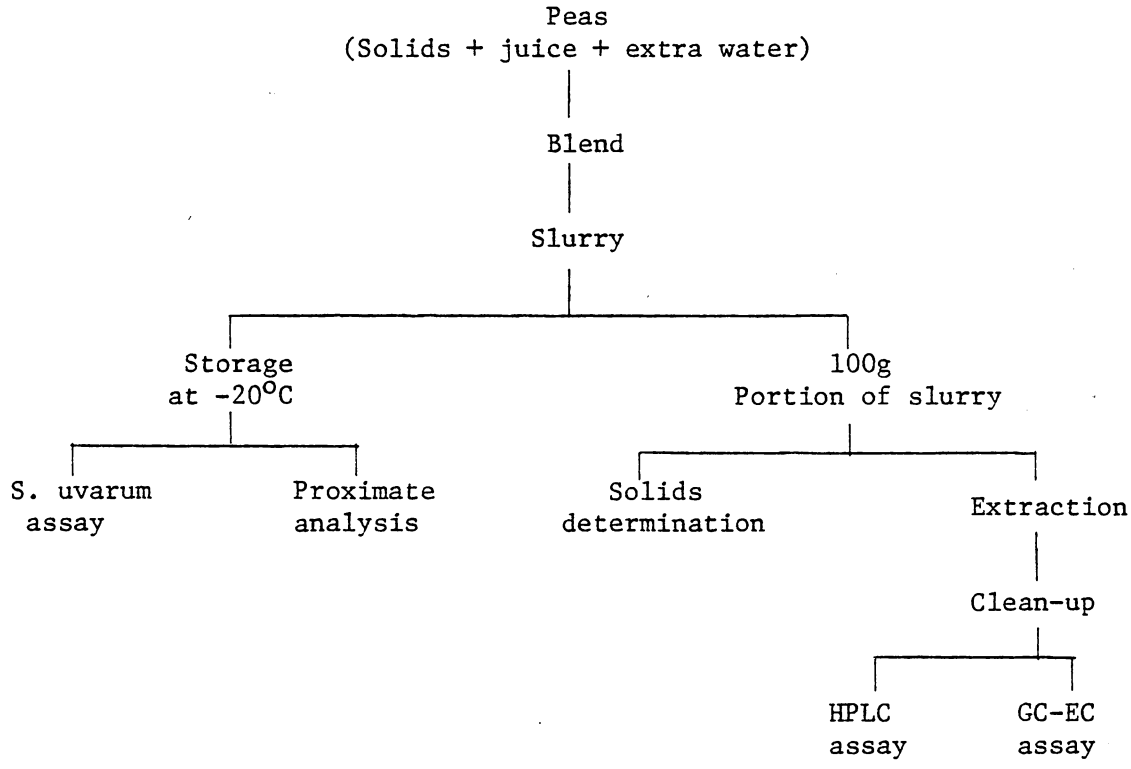


Figure 5 -- Preparation of pea sample

were then wrapped with aluminum foil. The pea slurry was stored in 500 ml wide-mouth amber glass bottles with black plastic screw-caps.

D. Determination of solids content of slurry

Fifty ml each of the bread and the milk slurry and 25 ml of the pea slurry were weighed. From the known weight of a given volume of slurry as well as the composition of the slurry (food: liquid ratio), the solids content (wet wt) of a known volume of slurry was computed as follows:

$$\text{Solids content (wet wt, g/ml slurry)} = \frac{\text{slurry wt} \times \frac{\text{wet wt food}}{\text{wet wt food + wt liquid added}}}{\text{slurry vol}}$$

The solids content of the different food slurries are shown in Table 2 and were used in calculating the contents of B_6 vitamers expressed as µg/100g wet wt of food.

E. Proximate analyses

Procedures for proximate composition determinations of the foods were essentially those of the Association of Official Analytical Chemists (61) with minor modifications.

1. Crude protein

The following sample sizes were used in the analyses: bread, 2.3g (wet wt); milk powder, 1.0g (wet wt); pea slurry, 1.0g (freeze-dried wt).

Table 2 -- Solids content of food slurries[a]

Food	Slurry Vendor	Solid content (g wet wt/ml slurry)
Bread	K*	0.29
	R**	0.37
Milk	K	0.31
	R	0.32
Peas	K	0.46
	R	0.42

[a] Mean of duplicate determinations.

* Kroger Co., Blacksburg, Virginia store.

** Radford Bros., Inc., Blacksburg, Virginia.

Nitrogen determinations were carried out by the macro-kjeldahl procedure (37) using 0.09006 N HCl for titration and a methyl red indicator. A general factor of 6.25 was used to calculate crude protein content.

2. Moisture

The following sample sizes were used: bread, 16g (wet wt); milk, 12g (wet wt) and pea slurry, 17g (wet wt). The samples were placed in a preweighed aluminum pans and dried in a drying oven at 75°C for 36 hr. The samples were then cooled for 2 hr in a dessicator and weighed. After the first weighing, the samples were dried again at 75°C for 3 hr, cooled for 2 hr, and reweighed. This last heat, cool, and weigh cycle was repeated 3 times until constant weights of samples were obtained. All weighings were done on a Mettler H-33 analytical balance.

3. Crude lipid

Clean labelled fat extraction beakers were dried in an air drying oven (80°C) for 24 hr and were cooled in a dessicator for approximately 6 hr. The beakers were weighed on a Mettler H-33 analytical balance and the weights recorded. Approximately 2 g of each of the freeze-dried food samples were transferred into fat extraction thimbles and placed on the Goldfisch fat extractor. Petroleum ether was used as the solvent for fat extractor. Petroleum ether was used as the solvent for fat extraction. The extraction was carried out for approximately 16 hr overnight. After reclaiming most of the petroleum ether and evaporating the remainder, the extraction beakers were placed in a dessicator for 24 hr. The beakers containing the extracted fat were weighed. The weights of the extracted fat were calculated by subtracting the original

weights of the beakers from the new weights of the beakers containing the extracted fat.

4. Ash

The following sample sizes were used: bread, 2.0 g (wet wt); milk, 2.0 g (wet wt); pea slurry, 3.0 g (wet wt, dried in oven at 100°C 2 hr prior to ashing). The samples for total ash were placed in 50 ml porcelain crucibles (Catalog No. 2371A, Fisher Scientific Co.) and heated in a muffle furnace at 400°C for 12 hr. The temperature of the furnace was then raised to 500°C for final ashing. The crucibles were cooled in a desiccator and weighed soon after room temperature was attained.

5. Carbohydrate

The carbohydrate content of the foods were calculated by subtracting the protein, fat, moisture and ash levels from 100%.

F. Extraction of B_6 vitamers from food slurries (see Fig. 7)

Extraction of the B_6 vitamers was carried out using the modified method of Wong (62). A mixture of 3 enzymes was used for the release of the B_6 vitamers bound to the food slurries. An alpha-amylase (1,4-alpha-D-Glucan glucanohydrolase; EC 3.2.1.1) was used for the digestion of starch and thus the release of any B_6 vitamers bound to the carbohydrate moiety in the food slurries. Pepsin (EC 3.4.23.1) was used to hydrolyze peptide linkages thus breaking down the native proteins of the food slurries into proteoses and peptones. Specific subtrates for pepsin include proteins, certain peptides and phosphoamides (63). Therefore,

pepsin was used to liberate any protein bound B_6 vitamers. Papain (EC 3.4.22.2) a vegetable pepsin which catalyzes the hydrolysis of proteins, proteoses, and peptones to amino acids was also used since it is active in acid, neutral or alkaline medium.

1. Preparation of enzyme solutions (Sigma Chemical Co., St. Louis, MO)

a. Alpha-amylase (Catalog No. A6630, 6%,w:w): Dissolve 3.0 g powdered enzyme in 50 g 2.5 M sodium acetate solution in a 100 ml beaker with mechanical stirring.

b. Pepsin (Catalog No. P7000, 6%,w:w): Dissolve 3.0 g powdered pepsin in 50 g 2.5 M Sodium acetate solution in 100 ml beaker with mechanical stirring.

c. Papain (Catalog No. P-3125, 10%,v:v): Dilute 1.0 ml papain solution to 10 ml with deionized-distilled water in a 10 ml volumetric flask. Shake to mix thoroughly.

2. Preparation of 0.2 N HCl

This was prepared by diluting 100 ml of 1.0 N Fisher prepared HCl solution (analytical grade) to 500 ml in a 500 ml volumetric flask with deionized-distilled water.

3. Extraction (see Table 3)

Volumetric flasks (100 ml each) were prepared with 5 ml of a spike solution (spike contains a mixture of pure hydrochloride salt of PAM, PAL, POL, AND DPOL). Fifty milliliters of slurry (bread or milk) or 25 ml of slurry (pea) were pipetted into a 100 ml volumetric flask using

Table 3 -- Extraction of B_6 vitamers in foods by acid hydrolysis in boiling water bath and enzyme digestion

	Bread 1:2[a] ml	Milk 1:2[a] ml	Peas 1:1[a] ml
Spike (PAM, PAL, POL, DPOL) or no spike (Just DPOL)	5.0	5.0	5.0
↓			
Slurry	50.0	50.0	25.0
↓			
0.2 N HCl	30.0	30.0	30.0
↓			
Add double distilled H_2O	0.0	0.0	25.0
↓			
(Boiling water bath)			
↓			
Enzyme mixture (α-amylase, pepsin, papain)	5.0	5.0	5.0
↓			
(Incubation)			
Final volume (adjust with double-distilled H_2O)	100	100	100

[a]Slurry composition (solids:liquid).

a 10 ml disposable bacteriological pipette. Each slurry was mechanically stirred while these aliquots were being withdrawn in order to ensure homogenous sampling. Thirty milliliters of 0.2N HCl was added to each flask, and those flasks containing pea slurry were adjusted to volume by the addition of 25 ml deionized-distilled water in order to obtain a total volume comparable to the other samples. This was done because the solid to liquid ratio for the pea slurry was 1:1 as compared to the ratio for the bread or milk slurry which was 1:2. Total composition of the mixtures in the flasks for acid hydrolysis and enzyme digestion are summarized in Table 3.

Acid hydrolysis of the samples was carried out in a boiling water bath for 1 hr. Constant stirring of the mixture was obtained with the use of a hot plate stirrer and placement of a magnetic stirring bar in each flask.

The flasks were cooled to room temperature and 2 ml alpha-amylase solution, 2 ml pepsin solution, and 1 ml papain solution were added to each flask - a total of 5 ml of composite enzyme mixture.

Samples were incubated at 37°C for 16 hr in a shaker water bath (Blue M Electric Co., Blue Island, IL) with the shaker set at No. 7 for medium speed shaking.

After incubation the magnetic stirring bars were removed from each flask and the content of each flask was adjusted to a final volume of 100 ml with deionized-distilled water and vacuum filtered through a No. 1 Whatman filter paper in a Buchner funnel.

G. Determination of best extraction method of B_6 vitamers from foods.

Utilizing the HPLC system developed for the detection of the 4 B_6 compounds POL, PAL, PAM and DPOL (See HPLC section), a preliminary experiment was conducted to determine the percent recovery of pure standards of POL, PAL, PAM, and DPOL which were subjected to 2 different extraction methods: 1) acid hydrolysis (0.1 N HCl) with autoclaving at 15 psig for 2 hr and 2) acid hydrolysis (0.1 N HCl) in boiling water bath followed by enzymatic digestion (see Fig. 6). Detection and quantitation of the B_6 standards after treatment by HPLC indicated a lower percent recovery of all 4 B_6 forms in the sample subjected to acid hydrolysis and autoclaving as compared to the sample subjected to acid hydrolysis in boiling water bath followed by enzymatic digestion (see Table 4).

Although vitamin B_6 has been reported as being stable to heat (64, 65), the data obtained in this study indicated a destruction of 14 to 17% of the 4 B_6 compounds subjected to autoclaving as compared to a loss of 0.5 to 5% of the same standard mixture of the 4 B_6 compounds subjected to the alternate extraction method (acid hydrolysis in boiling water bath followed by enzymatic digestion).

In view of these findings the extraction method involving acid hydrolysis in boiling water bath followed by enzymatic digestion was used in this study for the liberation of the B_6 vitamers from foods.

H. Removal of interfering substances from the food extracts

1. Preparation of 0.05N KOH

a. Potassium hydroxide solids (6.43g) were dissolved in 100 ml boiled

Table 4 -- Comparison of % recovery of B_6 standards from two different extraction treatments measured by HPLC

	Recovery (%)			
Extraction treatment	PAM	PAL	POL	DPOL
Acid hydrolysis with autoclaving	85.9	87.4	83.4	83.0
Acid hydrolysis with boiling water bath and enzyme digestion	97.0	99.5	95.0	96.1

Control	AOAC Method	Acid Hydrolysis Enzyme Digestion
2 ml mixture[a]	2 ml mixture[a]	2 ml mixture[a]
+	+	+
25 ml dist. deionized water	25 ml dist. deionized water	25 ml dist. deionized water
↓	↓	↓
60 ml 0.1 N HCl	60 ml 0.1 N HCl	60 ml 0.1 N HCl
↓	↓	↓
-	Autoclave 2 hr at 15 psig	Acid hydrolysis in boiling water bath
↓	↓	↓
-	Cool to room temperature	Cool to room temperature. Add 5 ml enzyme mixture, incubate for 2 hr at 47°C
↓	↓	↓
Filter through 0.45 µm millipore filter	Filter through 0.45 µm millipore filter	Filter through 0.45 µm millipore filter
↓	↓	↓
inject 20 µl into HPLC	inject 20 µl into HPLC	inject 20 µl into HPLC
↓	↓	↓
a ng	b ng	c ng
	% recovery = $\frac{a-b}{a}$ x 100	% recovery = $\frac{a-c}{a}$ x 100

[a]Mixture of PAM, PAL, POL and DPOL at a concentration of 600 ng/20 µl each.

Figure 6 -- Procedure to determine percent recovery of B_6 compounds after treatment by two extraction methods

deionized-distilled water to yield a stock solution of approximately 1 N KOH.

b. This stock solution was titrated against 25 ml of a 1.0N HCl standard solution with phenolphthalein as indicator.

c. The actual normality of the stock solution found from titration with the 1.0 N HCl standard was used to calculate the exact volume of the stock solution needed to make 1 L of 0.05 N KOH solution.

2. Preparation of Bio-Rad's AG-50W x 8 (100-200 mesh) resins in the K^+ form.

a. Three hundred grams of Bio-Rad's AC-50W x 8 (100-200 mesh) analytical grade cation exchange resin (Catalog No. 142-1441, Bio-Rad Lab., Richmond, CA) was rinsed 3 times with deionized-distilled water.

b. Six hundred milliliters of water was added to the rinsed resin in a 2 L beaker. With a Pasteur pipette 18 drops of phenolphthalein was added to the mixture.

c. The mixture was then mechanically stirred and titrated against a 6.0 N KOH solution until endpoint was reached. Titration was stopped when the supernatant of the mixture turned a permanent light pink.

d. After allowing the resin to settle, the supernatant was decanted and the resin was then rinsed 3 times with deionized-distilled water.

e. Nine hundred milliliters of 3.0 N HCl was added to the resins and the mixture heated in a boiling water bath for 0.5 hr with mechanical stirring. This process was repeated once.

f. After the resin had settled the supernatant was decanted and the resin was rinsed with deionized-distilled water until the rinse water

was neutral as indicated by a pH meter.

g. Six hundred milliliters of deionized-distilled water was added and step 3 was repeated.

h. After decanting off the supernatant the resin was rinsed with deionized-distilled water until the rinse water was neutral.

i. The resin was then suspended in about 360 ml 2 M potassium acetate solution and stored at 4°C until used.

3. Preparation of the chromatographic columns

a. Five to ten milliliters of water was poured into a chromatography column (400 mm x 19 mm I.D.) with a 250 ml reservoir (Catalog No. K-420290, Kontes, Vineland, NJ) with the teflon plug closed.

b. A glass wool plug was placed at the bottom of the column and bubbles from the column and glass wood were removed with the aid of a long glass rod.

c. About 30 ml of the prepared resin was rinsed with deionized-distilled water until the rinse water was neutral.

d. The resin was allowed to settle and excess water was decanted until a thick slurry of resin remained in the beaker.

e. 7.5 g of the slurry were poured into the column with the help of a stream of water from a wash bottle. A funnel with its tip against the side of the column was used in order to avoid formation of air pockets in the column during filling.

f. The resin was allowed to settle and a glass wool plug was placed on top of the resin.

4. Clean-up of food extracts

a. 25 ml aliquots of each of the filtered food extracts were pipetted into separate prepared chromatography columns and each column was then washed with 200 ml of deionized-distilled water.

b. The wash waters were monitored in four 50 ml portions using the HPLC method but no B_6 vitamers were detected.

c. The B_6 vitamers were eluted with 0.05 N potassium hydroxide solutions at a flow rate of approximately 20 ml/min and 75 ml of the eluate were collected in a measuring cylinder (it had been determined in a prior experiment with standards that the eluate collected after the initial 75 ml did not contain any B_6 vitamers.)

d. 0.45 μM filter utilizing a Swinnex type syringe filter (Millipore Corp., Bedford, MA) prior to injection into the HPLC system and to derivatization for GC-EC analysis.

5. Percent recovery of standards

A preliminary experiment was performed to determine the percent recovery of the 4 B_6 compounds from clean-up treatment using standards. A standard solution of a mixture of the 4 pure B_6 compounds with a concentration of 100 ng/20 μℓ for each compound was subjected to the clean-up treatment using Bio-Rad's AG 50W x 8 in K^+ form. The percent recovery of each B_6 compound was determined by comparing the concentration of each B_6 compound recovered in each 25 ml eluate using 0.05 N potassium hydroxide with the concentration of the standard solution (100 ng/20 μℓ). Table 3 shows the percent recovery of each B_6 compound in the first, second, and third 25 ml eluate from the chromatography

Table 5 -- Percent recovery of vitamin B_6 standards[a] after removal of interfering substances

Eluate	PAM	PAL	POL	DPOL
	Recovery (%)			
1st 25 ml	58.2	46.7	62.9	58.5
2nd 25 ml	15.2	27.4	25.5	25.0
3rd 25 ml	1.8	3.1	2.4	2.5
Total percent recovery for 75 ml	75.3	77.2	90.9	86.0

[a] standard solution of a mixture of PAM, PAL, POL and DPOL at a concentration of 100 ng/20 µl each.

column. No B_6 compounds were detected in eluates collected after the initial 75 ml eluate by HPLC. Total percent recoveries for PAM, PAL, POL, and DPOL were 75.3, 77.2, 90.9, and 86.0 respectively.

HPLC assay for B_6 vitamers in food extracts

A. Method

1. Analytical system

The 1 L bottle containing the mobile phase was set on a magnetic stirrer to provide for mechanical stirring during runs. This constituted the mobile phase reservoir. The HPLC apparatus (see Fig. 7) was constructed from commercial components including a model ALC 201 chromatography pump, a model 440 UV absorbance detector (Waters Associates, Milford, MA), and a model 7125 syringe loading sample injector with a 20 µℓ fixed-volume loop (Rheodyne Incorporated, Berkeley, CA). The analytical column was a Spherisorb ODS 25 cm x 4.6 mm i.d. 10 µm particle column obtained from Laboratory Data Control (Riviera Beach, FL) and fitted with a guard column packed with Co:Pell ODS (Whatman, Clifton, NJ). Recording was done with a Fisher Recordall model 5000 recorder (Fisher Scientific Co., Pittsburgh, PA).

2. Reagents

Acetonitrile was glass-distilled HPLC grade (Burdick & Jackson Lab, Muskegon, MI). The B_6 vitamer standards used were hydrochloride forms of POL, PAL, PAM, and DPOL (Sigma Chemical Co., St. Louis, MO). All other reagents were of analytical reagent grade.

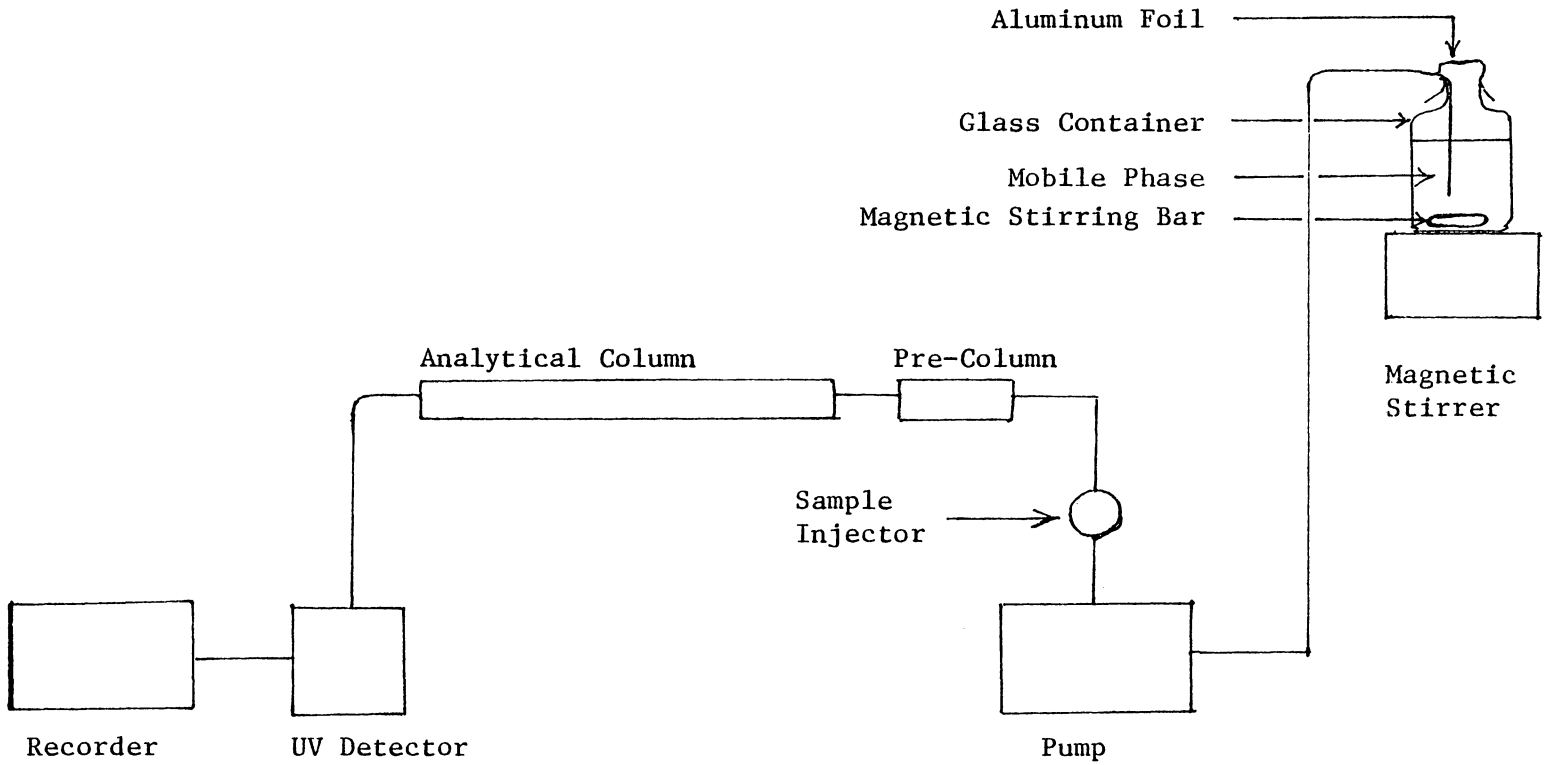


Figure 7 -- HPLC analytical system

3. Mobile phase

a. Approximately 1 L of deionized-distilled water was acidified with orthophosphoric acid to about pH 2.2 (pH meter, and mechanical stirring).

b. Nine hundred milliliters of the prepared acidified water was added to a 1 L volumetric flask containing 4.4910 g of potassium phosphate, monobasic and a solution obtained by repeated inversion of the volumetric flask until all the potassium phosphate was dissolved.

c. The potassium phosphate solution was then transferred into a 2 L beaker and the pH of the solution was readjusted to pH 2.2 with additional orthophosphoric acid.

d. The solution was poured back into the 1 L volumetric flask and brought to volume with the acidified water (pH 2.2).

e. A 5 ml aliquot of the homogeneous 0.033 M potassium phosphate buffer solution was checked with a pH meter to ascertain a final pH of 2.20 ± 0.01 for the solution.

f. The solution was then vacuum-filtered through a 0.45 μM filter (Millipore Corporation, Bedford, MA) in a Buchner funnel.

g. Into a separate 1 L volumetric flask was introduced 10 ml of prefiltered acetonitrile (Burdick and Jackson Labs, Muskegon, MI). Filtered potassium phosphate solution was then added to the acetonitrile and the mixture brought to volume to yield a mobile phase of 1:99, v:v, composition of acetonitrile to phosphate buffer. The mobile phase was stored at 5°C in a reagent bottle when not used. Prior to use the mobile phase was contained in a 1 L glass bottle which was immersed in hot water (70°C) for 5 min and then degassed with a vacuum outlet for

1.5 min while being mechanically stirred.

4. Experiment to determine the composition of the mobile phase

A preliminary experiment was carried out to determine the composition of the mobile phase which would permit the best resolution of the 4 B_6 forms by varying the concentration of acetonitrile and water. The following volume to volume ratios of acetonitrile to water were used: 50:50, 40:60, 30:70, 20:80, 10:90, 5:95; 3:97; 1:99. The 1:99, v:v, ratio of acetonitrile to water was found to give the best resolution of all 4 B_6 forms under the HPLC operating conditions of this study.

B. External standardization

1. Preparation of standards

a. The hydrochloride forms of PAL (0.0609 g), POL (0.0608 g), PAM (0.0717 g), and DPOL (0.0619 g) were weighed into a 100 ml volumetric flask and brought to volume with deionized-distilled water to yield a standard solution to 10^4 ng/20 µℓ of each of the free compounds (the concentration was based on 20 µℓ since the injector is equipped with an injection loop of a fixed volume of 20 µℓ).

b. Serial dilutions of the original stock solution (10^4 ng/20µℓ) were then carried out to obtain subsequent standard solutions with the following concentrations of the four compounds: 10^3 ng/20 µℓ, 500 ng/20 µℓ, 250 ng/20 µℓ, 100 ng/20 µℓ, 50 ng/20 µℓ, 25 ng/20 µℓ, 10 ng/20 µℓ, and 5 ng/20 µℓ.

c. Preparation of the original stock standard solution (10^4 ng/20 µℓ) and all serial dilutions were made in duplicate. The standards were protected from light and stored at 5°C when not in use.

2. Calibration

All solutions were filtered through a 0.45 µM millipor filter using a Swinnex type syringe filter and 2 ml of the filtrate were injected into the HPLC using a 5 ml Plastipak disposable syringe equipped with a Rheodyne No. 7125 stainless steel injection needle (Rheodyne Incorporated, Berkeley, CA). The injection loop was always flushed twice with filtered deionized-distilled water before a new injection was attempted.

3. Operating conditions

The mobile phase was pumped at a flow-rate of 1 ml/min at 1000 p.s.i.g at ambient temperature. The UV-detector was set at 280 nm with an attenuation varying from x0.005 to 2.0 for the whole range of concentration of standard mixtures (see Table 6). The recorder was set at 10 mV with a chart speed of 2 cm/min.

C. Internal standardization

A preliminary experiment was conducted to determine the relative response factors of the vitamers at different concentration ratios of the B_6 vitamers to DPOL, the internal standard. The actual concentrations used to obtain a blend of vitamers to DPOL in the ratios of 1:2, 1:1, and 2:1 are shown in Table 7. The detector attenuator was manually adjusted as needed during the course of each run so that the chromatograms

Table 6 -- Concentrations of standard solution of B_6 compounds calibrated at different detector attenuation setting

B_6 compounds (ng/20 µl)	UV-Detector Attenuation setting
5.0	0.005
10.0	0.005
25.0	0.005
50.0	0.01*
100.0	0.02
250.0	0.05
500.0	0.1
1000.0	0.2
10000.0	2.0

* All calculations for peak heights are based on an attenuation of 0.01.

Table 7 -- Relative response factors (RRF) of the B_6 vitamers at different concentration ratios of the vitamers to DPOL

Vitamers:DPOL (ng/20 µl) (ng/20 µl)	Attenuation	RRF PAM[a]	RRF PAL[a]	RRF POL[a]
5:10	0.005			
5:5	0.005	3.32 ±0.22	2.73 ±0.08	1.49 ±0.09
10:5	0.005			
5:10	0.01			
5:5	0.01	3.37 ±0.15	2.81 ±0.04	1.50 ±0.07
10:5	0.01			
20:40	0.01			
20:20	0.01	3.25 ±0.28	2.80 ±0.14	1.49 ±0.08
20:20	0.01			
50:100	0.01			
50:50	0.01	3.08 ±0.10	2.63 ±0.08	1.45 ±0.05
100:50	0.02			
100:200	0.05			
100:100	0.05	3.33 ±0.09	2.77 ±0.09	1.49 ±0.05
200:100	0.05			
	Average[b]	3.27 ±0.20	2.75 ±0.11	1.48 ±0.07

[a] Average of 6 chromatographic runs.

[b] Average of 30 chromatographic runs.

could remain within the boundaries of the recorder chart.

D. Co-chromatography (spiking)

The procedure for adding DPOL and the standard solution of a mixture of B_6 vitamers (spiking) is shown in Fig. 8. 5 ml of DPOL standard solution of concentration 12 x 10^3 ng/20 µℓ was added to the food slurry in each flask at the beginning of the experiment. These were the unspiked samples. Five milliliters of a standard solution of a mixture of PAM, PAL, POL, and DPOL of concentration 12 x 10^3 ng/20 µℓ each were added to the food slurry in each flask for the spiked samples. The control flask contained 5 ml of the standard solution of a mixture of PAM, PAL, POL, and DPOL (12 x 10^3 ng/20 µℓ concentration each) but no food slurry. The control was not subjected to extraction and clean-up treatments but appropriate dilutions prior to injection were made as shown in Fig. 10.

E. Analysis of the food extracts

Twenty microliters of the prefiltered food extract from which interfering substances had been removed by column chromatography was injected into the HPLC machine. Conditions for the run were the same as those for external standardization except for the detector attenuation which was set at x.01 throughout the course of the experiment.

Duplicate blanks which contained enzymes from the extraction procedure were also monitored for possible B_6 vitamers originating from the enzyme mixture (alpha-amylase, pepsin, and papain) but no B_6

Blank Containing Enzymes	Unspiked	Spiked	Control
-	Food slurry	Food slurry	-
	↓	↓	
-	(+ DPOL)	(+ DPOL + B_6 vitamers)	(+ DPOL + B_6 vitamers)
	↓	↓	↓
Extraction	Extraction	Extraction	-
↓	↓	↓	↓
Adjust to 100 ml	Adjust to 100 ml	Adjust to 100 ml	Adjust to 100 ml
↓	↓	↓	↓
25 ml aliquot	25 ml aliquot	25 ml aliquot	25 ml aliquot
↓	↓	↓	↓
Clean-up	Clean-up	Clean-up	-
↓	↓	↓	↓
75 ml eluate	75 ml eluate	75 ml eluate	Adjust to 75 ml
↓	↓	↓	↓
20 µl injection for HPLC assay	20 µl injection for HPLC assay	20 µl injection for HPLC assay	20 µl injection for HPLC assay

$$\% \text{ recovery of } B_6 \text{ vitamers or DPOL} = \frac{(b) - (a)}{(c)} \times 100$$

Figure 8 -- Addition of internal standard (DPOL) and standard solution of B_6 vitamer and calculation of % recovery of B_6 vitamers after treatment

vitamers were detected.

GC-EC assay for B_6 vitamers in food extracts

A. Methods

1. Analytical system

Gas chromatography was carried out using a Microtek MT-220 (Microtek Instruments, Baton Rouge, LA) gas liquid chromatograph fitted with a ^{63}Ni electron capture detector. The detector voltage was set at 10^2 and the sensitivity at 1/32.

The column was 1.54 m x2 mm i.d. glass, packed with 10% SP 2100 on Supelcoport 80-100 mesh (Supelco Inc., Bellefonte, PA).

2. Reagents

The B_6 vitamer standards were hydrochloride forms of POL, PAL, PAM, and DPOL. N-methyl-bis-trifluoroacetamide (MBTFA) was used as the derivatizing reagent. MBTFA has been reported to trifluoroacylate primary and secondary amines, hydroxyl, and thio groups under mild, non-acidic conditions (66). Pesticide grade 100% ethanol and ethyl acetate were glass-distilled before use.

B. External standardization

1. Preparation of standards

Stock solutions of individual B_6 forms (100 ng/µℓ of each) and a mixture of all 4 forms (DPOL, POL, PAL and PAM) were prepared in deionized-distilled water and protected from light. The preparation

procedure of the vitamin B_6 standard solution was similar to that given previously for HPLC. Trifluoroacetylation of the B_6 forms was carried out using a modified method of Patzer and Hilker (35). A 50 µℓ volume of each of the aqueous solution of the vitamin B_6 forms were introduced into separate 1 ml reactivials and dried under a gentle stream of N_2 at 65°C with the use of a No. 18800 Reactitherm heating module equipped with a No. 18804 Reacti-block (pierce Chem. Co., Rockford, IL).

To each vial were added 50 µℓ of 100% ethanol in order to convert PAL to its hemiacetal to distinguish it from POL after derivatization (38). The vials were covered with teflon-silicone discs and sealed with open top screw caps. The contents of the vials were refluxed at 85°C for 30 min, cooled to room temperature, and then the ethanol was evaporated away under N_2 at 65°C. Fifty microliters of the acylating reagent MBTFA were added to each vial and refluxing with closed tops was carried out to 130°C for 20 min. The vials were allowed to cool to room temperature and 450 µℓ of ethyl acetate were added to bring about a 1/10 dilution of the derivatized mixture so that a concentration of 100 ng/µℓ of each vitamin B_6 form was obtained. The mixture was vortexed for 0.5 min to ensure homogeneity. The vials each containing 100 ng/µℓ of derivatized B_6 compounds were further diluted with ethyl acetate to obtain concentrations of 0.01, 0.05, 0.1, 0.5, 1.0, 5.0, and 100.0 ng/µℓ. A volume of 1.0 µℓ was then injected directly into the GC and a calibration curve was obtained.

2. Operating conditions

The column temperature was maintained at 125°C, the injection port was operated at 205°C and the detector temperature was set at 350°C.

The carrier gas was nitrogen with a regulator pressure of 40 p.s.i. g and a flow-rate of 30 ml/min.

C. Analysis of food extracts

A 150 µℓ aliquot of the prepared food extract was derivatized under the same conditions as for the B_6 standards. A calibration curve with external standards was always made to accompany each new chromatographic run of a food extract. This took into account the inconstancy of the detector sensitivity and other associated chromatograhic parameters in between runs. Additionally a check for the presence of B_6 vitamers in the enzymes used for the extraction process was also made with every new run. However, no B_6 vitamers were detected.

Microbiological assay for B_6 vitamers in food extracts

The S. uvarum assay was essentially that of the Official Methods of Analysis of the Association of Official Analytical Chemists (61) with minor modifications. An outline of the assay plan is shown in Fig. 9.

A. Preparation of samples

1. Freeze-drying

Approximately 30 g wet wt of bread, 20 g wet wt of nonfat dry milk, and 40 g of pea slurry were weighed into clean, dry and preweighed petri-

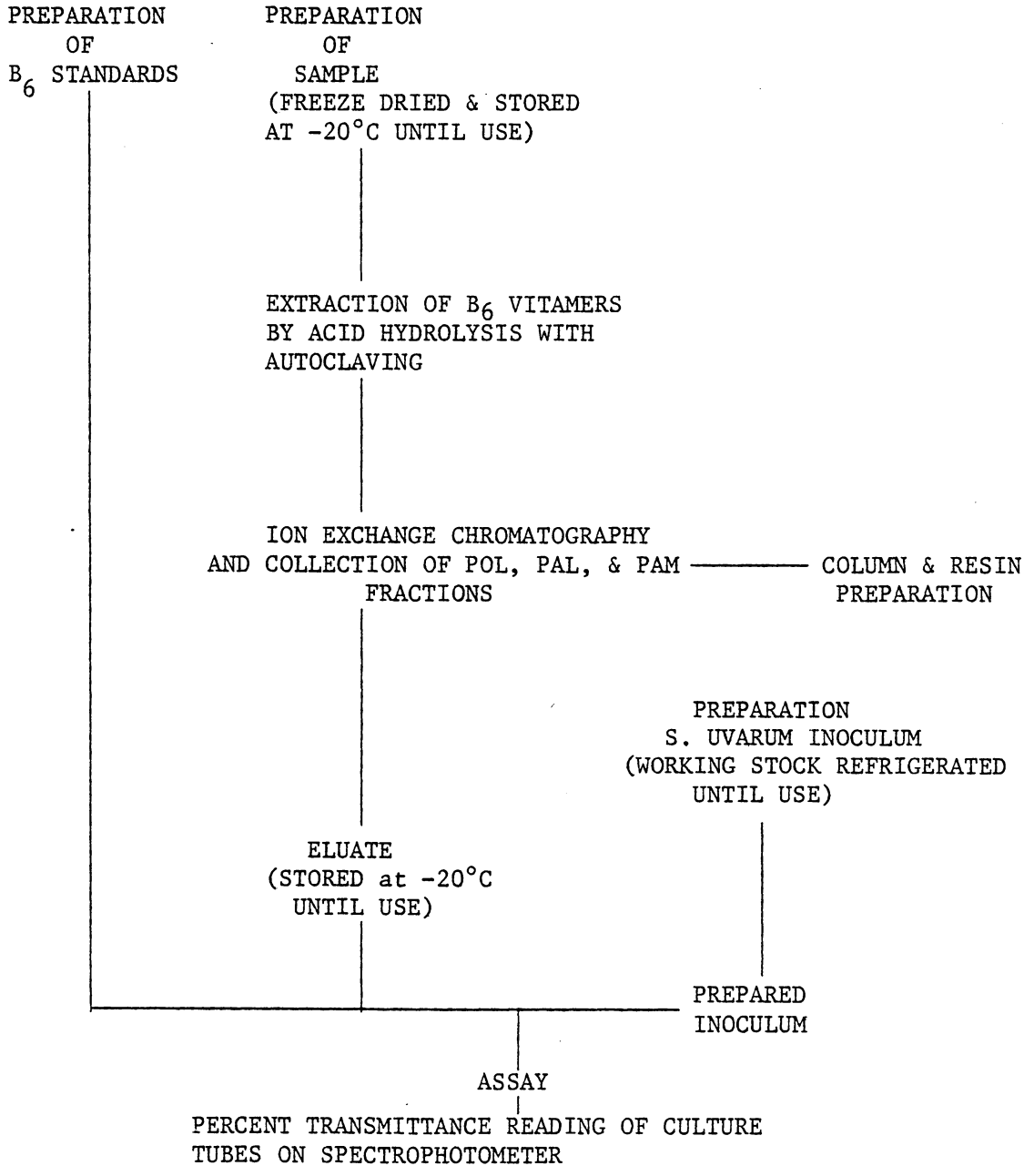


Fig. 9 -- Outline of Saccharomyces uvarum assay for B_6 vitamers in selected foods

dishes and the weight of each food plus container before freeze-drying was recorded. The foods were then freeze-dried for 48 hr and the wt of each food and container after freeze-drying was recorded. All weighings were carried out in duplicate. The recorded weights of the foods before and after freeze-drying were used to adjust the contents of the B_6 vitamers present in the food to an equivalent wt in µg per 100 g wet wt of the food.

2. Extraction of B_6 compounds from foods

The freeze-dried food, 2 g, was weighed into a 500 ml Erlenmeyer flask which was then covered with aluminum foil. For bread and peas 200 ml 0.44 N HCl were added to each sample and the solution autoclaved for 2 hr at 121°C. For milk, 200 ml 0.055 N HCl were added and the solution autoclaved for 5 hr at 121°C. After autoclaving the flasks were cooled to room temperature. The pH of each solution was adjusted to 4.5 with saturated potassium hydroxide and then diluted to 250 ml with water in a 250 ml volumetric flask. Each solution was then gravity filtered through Whatman No. 1 paper and the filtrate was collected in a 500 ml light protected actinic-glass Erlenmeyer flask which was then sealed with parafilm and frozen at -20°C until analyses.

B. Preparation of ion-exchange resin and column

Preparation of the ion-exchange resin and the chromatographic column for separation of the 3 B_6 vitamers has been previously described.

C. Chromatography of sample extract

Two hundred milliliters of the filtered extract were transferred into the ion-exchange column in 100 ml portions. The extract was allowed to pass through at a flow-rate of approximately 6 ml/min. Care was taken throughout the chromatographic procedure to ensure that the liquid level in the column did not fall below the surface of the resins. This was to prevent the formation of air bubbles or disturbance of the resin packing in the column.

The beaker and column were washed 3 times with about 5 ml portions of hot (70°C) 0.02 M potassium acetate (pH 5.5) followed by similar washings to column sides. Washing of the column with hot 0.02 M potassium acetate (pH 5.5) was continued until a total of 100 ml of the solution were used. Pyridoxal was eluted with two 50 ml portions of boiling 0.04 M potassium acetate (pH 6.0) using a 100 ml volumetric flask as a receiver. Pyridoxine was eluted with two 50 ml portions of boiling 0.1 M potassium acetate (pH 7.0) using a 100 ml volumetric flask as receiver. Pyridoxamine was eluted with two 50 ml portions boiling potassium chloride di-potassium hydrogen phosphate ($KCl-K_2HPO_4$) (pH 8.0) solution using a 200 ml volumetric flask as receiver. The pH of the pyridoxamine eluate was adjusted to pH 4.5 with 10% acetic acid. The pyridoxine and pyridoxal eluates were each diluted to 100 ml and the pyridoxamine eluate to 200 ml with distilled-deionized water.

D. Chromatograhy of B_6 standards

Ten milliliters of each intermediate solution (see Appendix 1) of pyridoxine, pyridoxal, and pyridoxamine were mixed in a 100 ml beaker.

The mixture was neutralized with dilute KOH solution and then adjusted to pH 4.5 with diluted acetic acid solution. The mixture was transformed in to an ion-exchange column prepared as above and washing and elution of each of the pyridoxal, pyridoxine and pyridoxamine fractions was carried out as in the chromatography of the sample extract. The eluted standards were each diluted to a concentration of 1.0 ug/ml with distilled-deionized water. This was carried out by diluting 1.0 ml of the pyridoxal eluate to 100 ml each. For the pyridoxamine eluate 2 ml of the eluate was diluted to 100 ml to yeild a concentration of 1.0 ng/ml. All eluates were kept at 5°C until ready for assay.

E. Preparation of assay inoculum

1. Preparation of Saccharomyces uvarum culture

Saccharomyces uvarum (ATCC No. 9080) was obtained from American Type Culture Collection (Rockville, MD). To grow the culture, 0.4 ml of liquid medium (YM broth-Difco 0711) was added to the freeze-dried culture using a disposable serological pipet.

After mixing well the total mixture was transferred to a test tube of 5 ml sterile (autoclaved) water and the freeze-dried test organism was allowed to soak for 30 min.

Six agar slants (YM Agar-Difco 0712) were labelled with the name of the organism and date. Using a loop each slant was inoculated with a streak spread over the entire surface from bottom to top. The freshly seeded agar slants were then incubated for 24 hr at 30°C in a Fisher Isotemp Incubator (Deluxe Model, Fisher Scientific Co., Pittsburgh,

PA). The tubes were then removed and 3 of them were labelled "reserved stock" while the other 3 were labelled "working stock."

The 6 tubes were refrigerated at 5°C until needed. The "reserve stock" was used for making weekly transfers to 3 new "reserve stock" slants and 3 new "working stock" slants. A gram stain and microscopic examination was made weekly so check the working stock culture for contamination (see Appendix 2 for gram stain procedure).

2. Preparation of the inoculum

Before use the 3 tubes of agar slant labelled "working stock" were incubated for 24 hr at 30°C. The cells were transferred under aseptic conditions (by flaming in between transfers) to 10 test tubes containing liquid culture media. The test tubes were then covered with plastic caps (Kim-Kaps, Fisher Scientific Co., Pittsburgh, PA) and incubated at 30°C for 20 hr in a shaker water bath (Blue M Electric Co., Blue Island, IL) with the shaker set at number 3 for gentle agitation.

After 20 hr the tubes were removed from the shaker water bath and the cells were centrifuged for 2 min at 300 G in an IEC size 2, model K Centrifuge (International Equipment Co., Needham Heights, MD). The liquid in each tube was decanted and the cells resuspended in 10 ml of sterile inoculum rinse. The process of centrifugation, decanting, and resuspension of cells in sterile inoculum rinse was repeated 3 times. The cells suspended in the final 10 ml inoculum rinse were used as the assay inoculum.

F. Analysis of the food extracts

One hundred and forty clean 16 x 150 mm disposable pyrex borosilicate glass culture tubes each containing two 4 mm glass beads were heated in an oven for 2 hr at 260°C. The tubes were cooled to room temperature and each tube was then covered with a sterile plastic cap.

For the standard curves, appropriate freshly prepared standard working solutions of concentration 1.0 ng/ml were pipetted into triplicate tubes to give 0.0, 0.0, 1.0, 2.0, 3.0, 4.0, and 5.0 ng of POL, PAL, and PAM per tube.

To obtain the curve of the eluted standards, 1.0, 2.0, 3.0, 4.0, and 5.0 ml of the eluted standards diluted to 1 ng vitamin B_6 components/ml were pipeted into triplicate tubes to give 1.0, 2.0, 3.0, 4.0, and 5.0 ng of POL, PAL, and PAM per tube.

Sample eluates from the chromatographic column were diluted to contain about 1 ng B_6 vitamer/ml. This was accomplished by making a 1:100 dilution for each of the bread and milk eluate and a 1:10 dilution for the pea eluate. Volumes of 1.0, 2.0, 3.0, 4.0, and 5.0 ml of each of the diluted sample eluates were pipetted into triplicate tubes.

Deionized-distilled water was pipetted into all tubes to bring the volume to 5 ml/tube. The tubes were recapped with the plastic caps and the entire set of standards, eluted standards and sample eluates was autoclaved at 121°C for 10 min. After autoclaving the tubes were allowed to cool to room temperature.

A Cornwall automatic pipet (Catalog No. P5173) attached to a 23 gauge x 2 inch Cannula (Catalog No. P5174-2) (Scientific Product Co.,

McGaw Park, IL) equipped with a sinker was sterilized by autoclaving at 121°C for 15 min. The sterilized pipet was used to deliver 5 ml steamed basal medium (Pyridoxine-Y-medium) under aseptic condition (flaming) into each of the tubes prepared above. The tubes were stored at 5°C in the refrigerator until analyses.

The tubes were removed from the refrigerator 1 hr before inoculation. Each tube (except the first set of 0.0 level blank for the standard curves) was aseptically inoculated with 1 drop of the assay inoculum of S. uvarum cells using a sterile short tip disposable pasteur pipet equipped with a rubber teat (Pasteur pipets were wrapped in aluminum foil and sterilized by autoclaving at 121°C for 10 min prior to assay). The test-tube containing the assay inoculum was gently vortexed after every 3 inoculations in order to maintain as uniform a concentration of cells as possible in each drop of inoculum used.

All the tubes were then incubated in a shaker bath (Model 75 Precision Scientific Co., Chicago, IL) at 30°C for 22 hr with shaker control set at about 45 oscillations per minute for gentle agitation. The tubes were then removed and autoclaved at 121°C for 5 min and cooled to room temperature.

Bausch and Lomb glass cuvettes were used to read the percent transmittance of each sample at 550 nm on a spectrophotometer (Spectronic 20, Bausch and Lomb, Rochester, New York). The uninoculated blank was used to set the instrument at 100% transmittance to read the inoculated blank. Then, the contents of 9 inoculated blank tubes were mixed and this mixture was used to set the instrument at 100% transmittance.

G. Calculation

1. Readings of duplicate tubes for standard working solution were averaged and percent transmittance was plotted against weight of standard POL, PAL, and PAM in ng per ml on semilog paper (unchromatographed B_6 standards, see Fig. 10).

2. Percent Transmittance readings of duplicate tubes for the eluted standards were averaged and the corresponding concentration of the vitamers were interpolated from the curve for the unchromatographed B_6 standards (see Fig. 11).

3. Percent Transmittance readings of duplicate tubes for the eluted extracts were averaged. The corresponding concentration of the vitamers were interpolated from the curve for the chromatographed B_6 standards and percent Transmittance was plotted against concentration (ng/ml) of POL, PAL, and PAM in the eluted sample extract.

4. Values of POL, PAL, and PAM were reported in µg/100 g wet wt.

Statistics

Statistical treatment of the data of the analysis included means and standard deviation. Correlations existing between the percent recovery of B_6 vitamers in foods and the percent crude protein content of the foods were calculated using Pearson's correlation coefficient, r (67, 68).

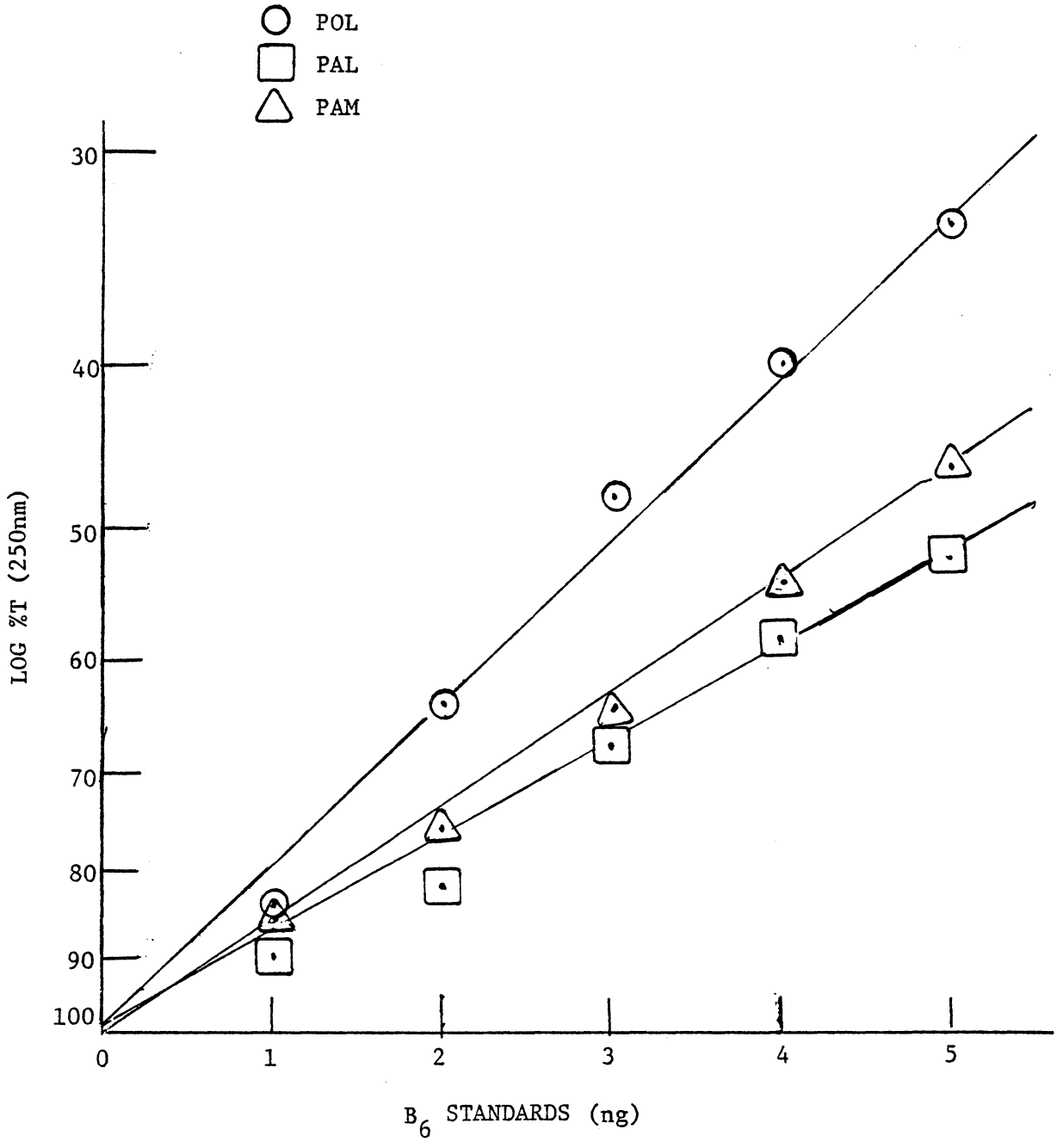


Fig. 10-- Chromatographed B_6 standards measured microbiologically

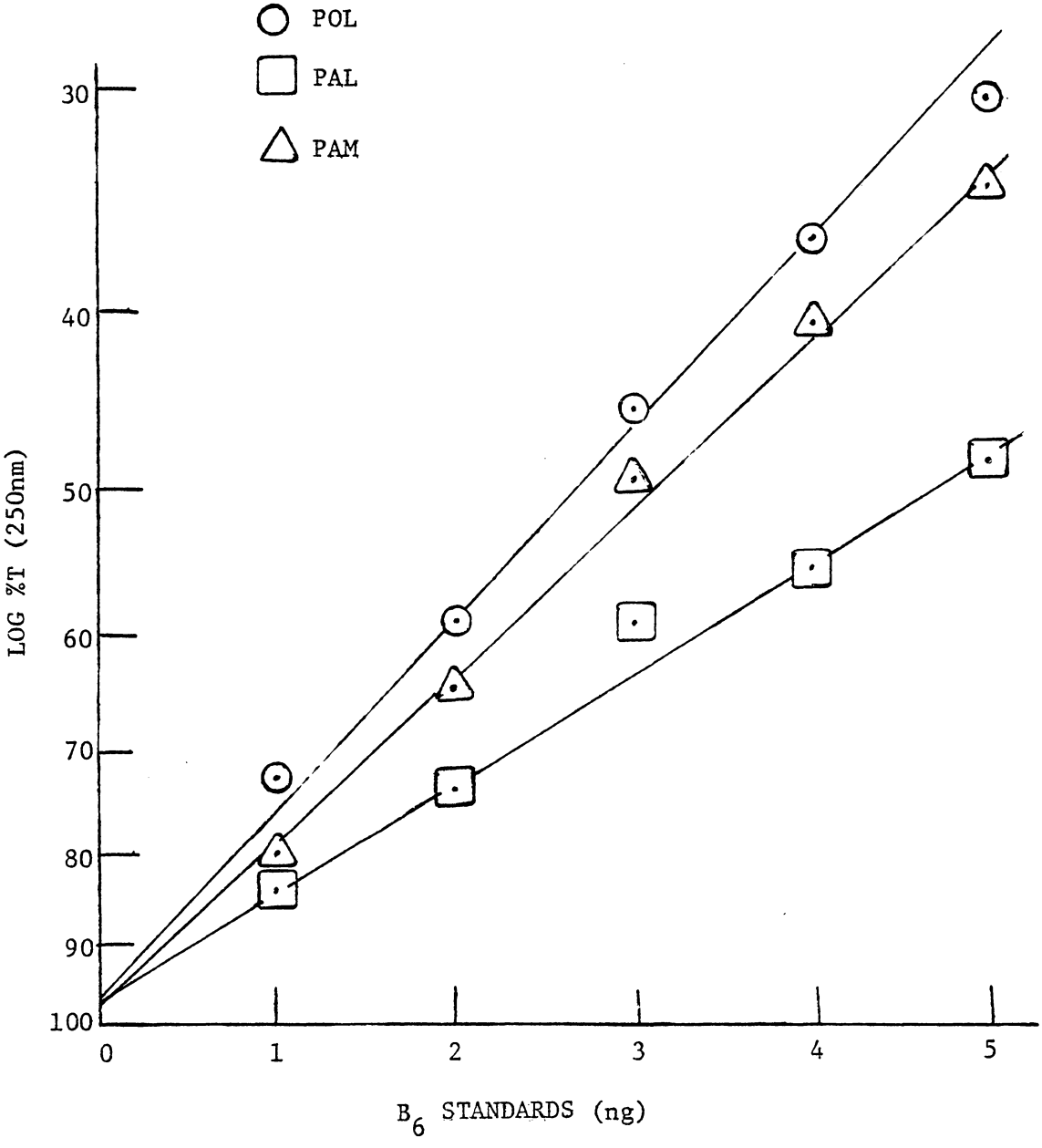


Fig. 11 -- Unchromatographed B_6 standards measured microbiologically

RESULTS AND DISCUSSION

The vitamin B_6 content of selected foods was determined by HPLC, GC-EC, and microbiological techniques. Proximate analyses were also performed on these foods.

Proximate analyses of foods

The proximate composition values for the 3 foods are shown in Table 8. In general the values were found to agree closely with values published in the Agriculture Handbook No. 456 (69) for similar foods.

HPLC analysis of B_6 vitamers in foods

The spherisorb:ODS column used was a reverse-phase column--i.e., the octadecylsilane stationary phase is non-polar, permitting chromatography in which the mobile phase is more polar than the stationary phase. The composition of the mobile phase, acetonitrile : water (1:99, v:v), was determined to yield peak patterns with the best resolution and least tailing. A reduction in the amount of acetonitrile resulted in excessive tailing whereas an increase in acetonitrile content over the ideal proportion of 1:99 gave poor peak resolutions especially between the PAL and POL peaks.

Table 8 -- Proximate analyses of selected foods

Food	Vendor	Moisture (%)	Crude lipid (%)	Crude protein (%)	Ash (%)	Carbohydrate by difference (%)
Bread	K	35.4	1.3	9.4	2.2	48.3
	R	35.7	1.7	9.6	2.1	50.9
	Average[a]	35.6	2.9	9.5	2.2	49.6
Milk	K	2.7	0.1	37.5	8.2	51.5
	R	3.3	0.1	33.8	7.8	55.0
	Average[a]	3.0	0.1	35.7	8.0	53.3
Peas	K	80.7	0.7	4.4	0.5	13.8
	R	77.6	0.7	5.4	0.7	15.6
	Average[a]	79.2	0.7	4.9	0.6	14.7

[a] Average of foods obtained from K and R.

A. External standardization

Good separation of the 4 B_6 compounds was obtained (Fig. 12). PAM was eluted first followed by PAL, POL, and DPOL. In HPLC work a separation time of less than 30 min is desirable (70). In this study all 3 B_6 vitamers were eluted in less than 10 min. With internal standardization, the separation time for all 4 B_6 compounds including the internal standard (DPOL) was less than 16 min.

The retention time of PAM was 3.7 min; PAL, 6.9 min; POL, 8.6 min; and DPOL, 14.1 min. The percent deviation of the retention time ranged from 0.66 to 1.25% (Table 9) indicating that high reproducibility of retention times for all the B_6 compounds was obtained with the HPLC system in this study. The retention time is the time required to elute a compound from a chromatography column (for both HPLC and GC) and is characteristic of the compound, column, solvent, and other associated operating conditions (70). Good reproducibility of the retention times of the B_6 compounds lends credence to the fact that this peak may correspond to the B_6 vitamer of interest. The retention times of PAL, POL, and DPOL can be expressed alternatively as retention ratios relative to PAM. From Table 5 the retention ratios of the other compounds (retention time of compound/retention time of PAM) relative to PAM were: PAL, 1.88; POL, 2.34; and DPOL,3.86.

A theoretical plate value of 1726.5 and a height equivalent to theoretical plate value of 0.014 cm for the 25 cm Spehrisorb:ODS column were obtained for the peak representing POL. Column efficiency can be measured quantitatively by its theoretical plate value, N

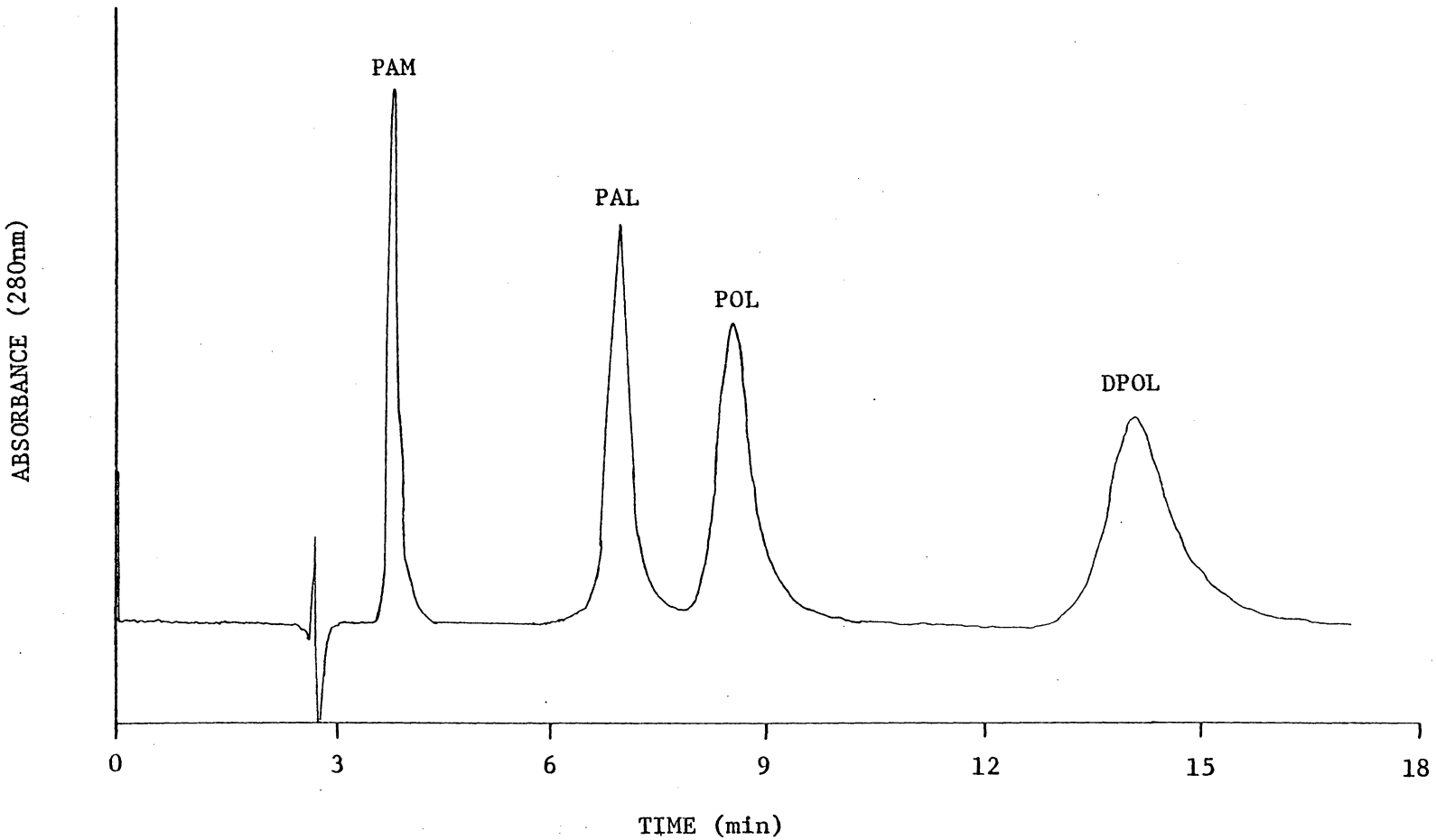


Fig. 12 -- Separation of B_6 standards by HPLC

Table 9 -- Reproducibility of retention times of B_6 compounds separated by HPLC

Run No.	PAM	PAL	POL	DPOL
	Retention times[a]			
1	74	138	169	281
2	74	138	169	280
3	74	138	170	282
4	74	137	170	282
5	74	140	174	284
6	73	139	173	283
7	72	139	172	285
8	72	139	174	286
Mean	73.37	138.50	171.37	282.87
Std. Dev.	±0.92	±0.92	±2.13	±2.03
% Dev.	1.25	0.66	1.24	0.72

[a] Retention time of each B_6 compound is expressed as the distance of its peak in millimeter from the chromatogram origin at a constant recorder chart speed of 2 cm/min.

[N = 16 (retention time/width at baseline)^2], which is proportional to column length or by the height equivalent to theoretical plate, H (H = column length/theoretical plate) which is a measure of column efficiency independent of column length. Columns for HPLC generally have plate heights in the range of 0.001 cm to 0.1 cm with better columns having smaller values for H (70).

A resolution, R, of 2.34 was calculated for the PAL and POL peaks, the only peaks not baseline resolved. R was computed from the following formula:

$$R = \frac{2(T_{POL} - T_{PAL})}{W_{POL} + W_{PAL}}$$

Where T_{POL} = retention time of POL

T_{PAL} = retention time of PAL

W_{POL} = width at baseline of peak corresponding to POL

W_{PAL} = width at baseline of peak corresponding to PAL

Typical calibration curves using external standardization for the 4 B_6 compounds are shown in Fig. 13; DPOL standards are also graphed. The calibration curves for all the B_6 forms were linear between 5 ng and 1000 ng. The peak heights of all the 4 B_6 compounds were expressed as their corresponding peak heights at the working attenuation (attn), x0.01, as follows:

$$\text{Peak ht at attn. 0.01} = \text{Peak ht at attn a} \times \frac{\text{MRF at attn 0.01}}{\text{MRF at attn a}}$$

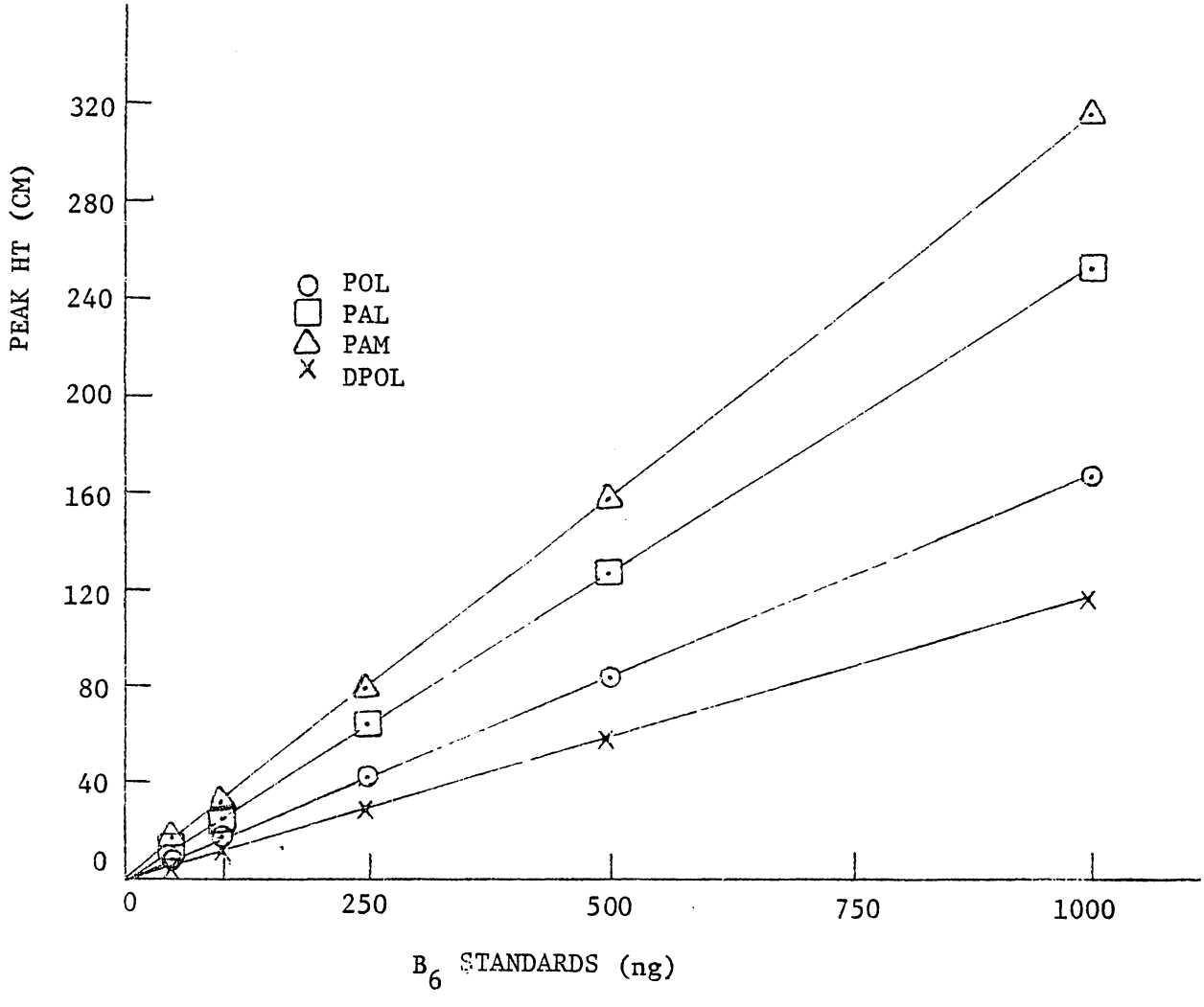


Fig. 13 -- B_6 standards measured by HPLC

Where a = attn setting other than 0.01

and MRF = mean response factor 9 i.e. slope of the calibration curve)

An inverse linear relationship was observed between the MRF and the attenuation setting (see Fig. 14) -- i.e., the MRF of a B_6 compound was function of the attenuation setting. Using data given in Fig. 13, the following mean response factors (Peak ht in mm : wt in ng) were obtained: PAM, 3.15; PAL, 2.51; POL, 1.64; and DPOL, 1.17.

B. Analyses of food extracts by external standardization

Good separation of the B_6 compounds in bread, milk, and pea extracts were obtained as shown in Fig. 15-17. Some interfering peaks were observed. However this was a significant improvement over chromatographic patterns as obtained by direct injections of extracts. No peaks corresponding to PAM were detected in the bread or pea chromatograms. All the 3 B_6 vitamers were detected in the milk extract as shown by its chromatogram (FIg. 15). The B_6 compounds were identified by co-chromatography (spiking) with the pure standards and by their retention times. The possibility that different compounds may have identical retention times is recognized. Therefore identification of the B_6 compounds by mass-spectrometry was attempted by collecting timed fractions of the effluent from the HPLC column of each B_6 compound in the food extracts in 5 ml glass vials and drying them at $60^{o}C$ with nitrogen gas. The residues were subjected

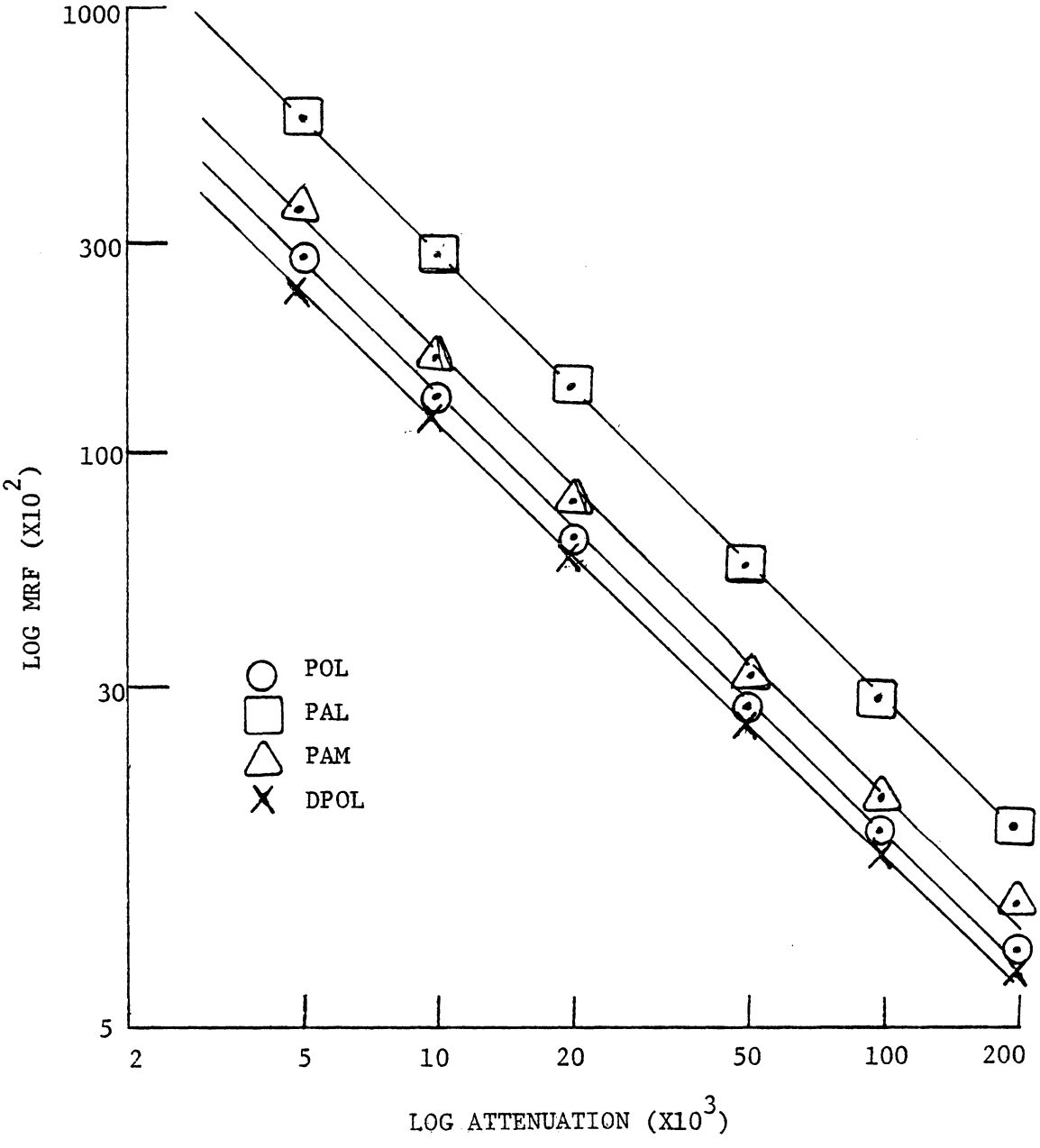


Fig. 14 -- MRF of B_6 standards versus attenuation of UV detector as measured by HPLC

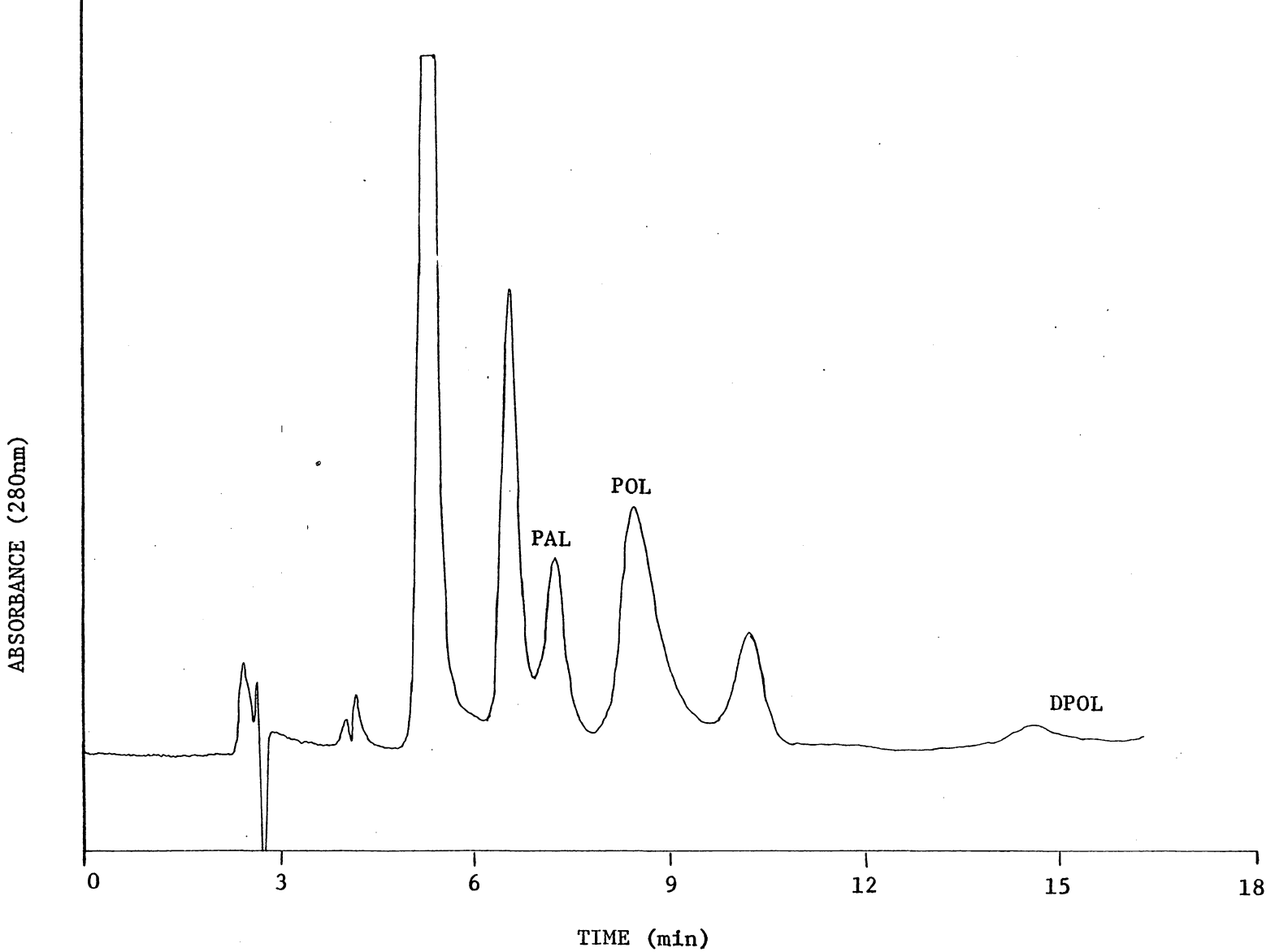


Fig. 15-- Separation of B_6 forms in bread by HPLC

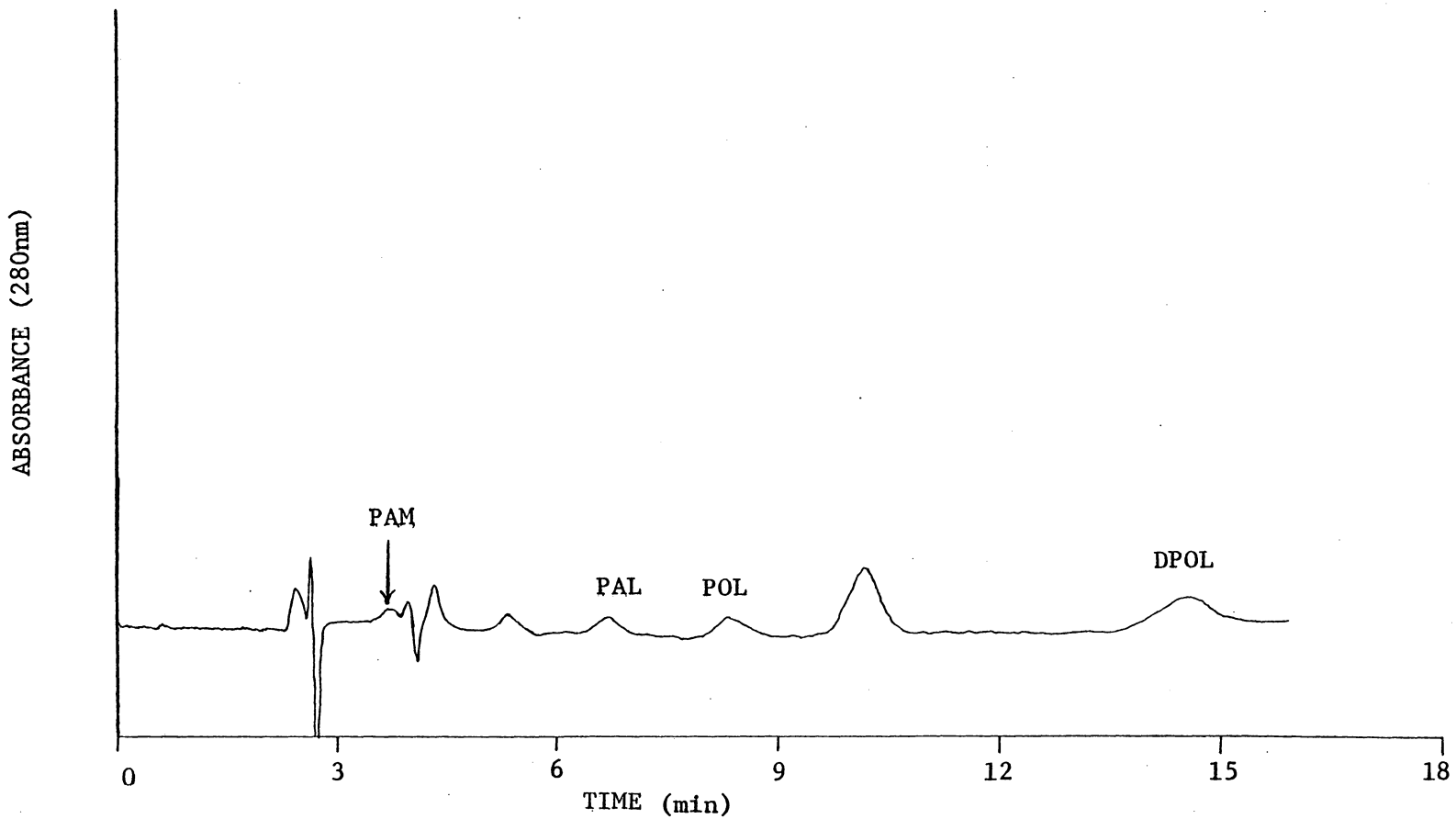


Fig. 16-- Separation of B_6 forms in milk by HPLC

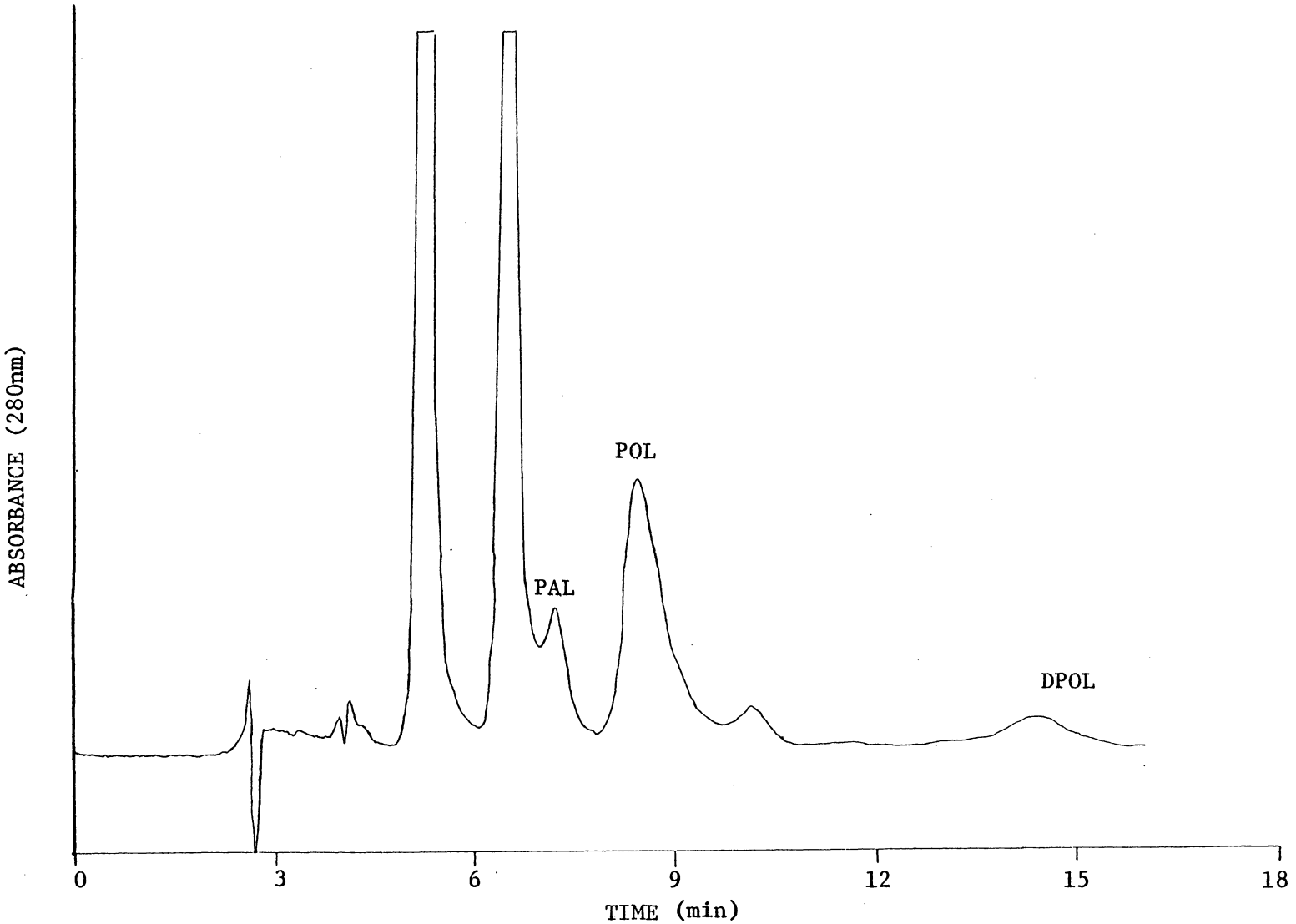


Fig.17-- Separation of B_6 forms in peas by HPLC

to mass-spectrometry using a "direct-probe" technique. Identification of the B_6 compounds by mass-spectrometry was not successful. This may have been due to the "dilution effect" of the HPLC solvent which did not permit a large enough sample to be collected for analysis by the mass-spectrometry system used which had a lower limit of sensitivity in the low µg ranges.

In order to resolve the real possibility of a coincidence of retention times for the particular sample it is suggested that future studies include investigations into minimizing the possiblity of errors by using auxiliary techniques such as mass-spectrometry and infrared-spectroscopy to identify the B_6 compounds.

C. Calculation of the B_6 vitamer content in foods using external standardization

The heights of peaks corresponding to the B_6 vitamers were measured from the chromatograms of the food extracts and the quantity of each vitamer in the corresponding food was calculated using the following formula:

$$\frac{\text{µg vitamer}}{\text{100 g wet wt food}} = \frac{1500 \times a}{MRF \times N}$$

Where 1500 = total dilution factor

a = peak ht of vitamer in mm

MRF = mean response factor of vitamer in mm/ng

N = wet wt of solids in g of a given volume of food slurry

The B_6 vitamer contents in bread, milk, and peas as obtained using external standardization are shown in Table 10 and the individual B_6 vitamer values are given in Appendix 3. It is speculated that the differences in the values of total vitamin B_6 for K and R, although small, may be a reflection of actual differences in different vitamer contents in foods from 2 different retail sources. The average values for total vitamin B_6 in each food may thus include variations due to source, instrument, as well as treatment and shelf-life.

The total vitamin B_6 value for milk as determined by HPLC in this study (641.9 µg/100g wet wt) was found to be in close agreement with the total vitamin B_6 value for non-fat dried milk (769.7 µg/100 g wet wt) as obtained by Vanderslice et al. (57).

D. Calculation of B_6 vitamer content in foods using internal standardization

A sample calculation of the B_6 vitamer content in the foods using internal standardization is shown in Table 11. A calibration mixture containing known amounts of PAM, PAL, and POL plus the internal standard DPOL was prepared and chromatographed (see column A, Table 11). For each peak the measured height was divided by the concentration of the associated vitamin B_6 compound to obtain a response factor. Each of the response factors were divided by that of the internal standard to obtain relative response factors. For each sample peak (see column B of Fig. 11) the measured height was divided by the proper relative response factor to obtain the corrected height. This step removed the effect of the detector non-uniformity. The corrected height was

Table 10 -- HPLC assay of B_6 vitamers in selected foods using external standardization[a]

Food	Vendor	PAM	PAL	POL	Total B_6
		(µg/100g wet wt)			
Bread	K	0.0[b]	1972.1[b] ±452.7	4188.3[b] ±502.0	6160.3[b] ±954.6
	R	0.0	1601.6 ±448.5	2154.9 ±518.2	3756.5 ±966.6
	Average[c]	0.0	1786.9 ±425.6	3171.6 ±1245.7	4958.5 ±1594.2
Milk	K	69.0 ±10.8	158.8 ±47.6	382.3 ±41.6	610.0 ±16.9
	R	88.8 ±0.0	223.0 ±13.1	362.0 ±110.4	673.7 ±97.2
	Average[c]	78.7 ±12.8	190.9 ±46.8	372.1 ±69.1	641.9 ±67.8
Peas	K	0.5 ±0.6	2218.7 ±55.0	5728.7 ±925.1	7947.4 ±870.0
	R	0.0	2360.0 ±79.5	5686.2 ±30.3	8046.2 ±109.8
	Average[c]	0.2 ±0.5	2289.3 ±98.8	5707.5 ±534.9	7996.8 ±509.5

[a] Values not corrected for % recovery.

[b] $\bar{X} \pm$ SD for duplicate analyses on two separate extractions.

[c] $\bar{X} \pm$ SD for foods obtained from K and R.

Table 11 -- Calculation of B_6 vitamer contents in food using internal standardization

A. Calculation for the calibrating standards

Pk	(mm)	Concentration (ng/20 µl)	RF[a] (Ht/conc.)	RRF[b] (RF/RF_{DPOL})
POL	16.00	5.0	3.2	1.6
DPOL	20.00	10.0	2.0	1.0

B. Calculation steps for the sample

Pk	(mm)	RRF	Corrected Ht. (Ht/RRF, mm)	Relative Amt. (Corr. Ht/Ht_{DPOL})	Actual Amt.[c] (µg/100g wet wt.)
POL	6.0	1.6	3.7	0.4	354.5
DPOL	8.5	1.0	8.5	1.0	-

[a] RF = Response factor in mm/ng.

[b] RRF = Relative response factor.

[c] Actual amount = $\frac{1500 \times \text{Relative Amt.} \times W_{DPOL}}{N}$

where 1500 = dilution factor.

W_{DPOL} = actual quantity of DPOL in a 20 µl injection, in this case 8.33 ng.

N = wet wt. of solids in g of a given volume of food slurry, in this case 15.51g.

divided by that of the internal standard to obtain the amount of each component relative to the internal standard. The actual amounts of each vitamer in μg present in 100 g wet wt of food was obtained by multiplying the relative amounts by a factor which included the known or actual quantity of the internal standard in a 20 μℓ injection, a dilution factor, and the wet weight of solids in the original volume of food slurry used in the analysis.

The vitamin B_6 content of bread, milk, and peas as calculated using the internal standardization method of calibration is shown in Table 12. The associated individual values used to generate Table 12 are in Appendix 4. Using information from Table 10 and 8 a table of percent relative difference between the mean values of each vitamer obtained by the internal standardization and the external standardization techniques was calculated (Table 13). On the whole the mean vitamer values calculated by the internal standardization are slightly larger than corresponding values calculated by external standardization as indicated by the predominance of positive percent relative difference given in Table 13. The percent coefficient of variation of mean B_6 vitamer values as calculated by both methods of calibration was also compared (Appendix 5). In general, the percent coefficient values associated with internal standardization are lower than those associated with the external standardization technique indicating somewhat better repeatability or precision with the internal standardization method.

The use of the internal standard DPOL, in this study tended to minimize errors resulting from sample preparation, apparatus, and

Table 12 -- HPLC assay of B_6 vitamers in selected foods using internal standardization[a]

Food	Vendor	PAM	PAL	POL	Total B_6
		(µg/100g wet wt)			
Bread	K	0.0[b]	2144.6[b] ±488.8	5538.0[b] ±666.4	7682.7[b] ±1155.3
	R	0.0	1854.5 ±322.4	3045.7 ±409.1	4900.3 ±731.4
	Average[c]	0.0	1999.6 ±377.3	4291.9 ±1508.1	6291.5 ±1789.9
Milk	K	63.9 ±11.0	140.4 ±44.8	409.1 ±36.9	613.4 ±18.9
	R	98.2 ±28.8	235.0 ±83.7	438.9 ±1.3	772.1 ±111.2
	Average[c]	81.1 ±26.6	187.7 ±77.4	424.0 ±27.4	692.8 ±112.4
Peas	K	0.5 ±0.7	2021.0 ±384.6	6239.3 ±34.1	8260.9 ±419.4
	R	0.0	2771.9 ±242.7	8115.8 ±485.3	10887.7 ±728.0
	Average[c]	0.3 ±0.5	2396.5 ±506.8	7177.6 ±1119.2	9574.3 ±1592.3

[a] Values not corrected for % recovery.

[b] $\bar{X} \pm$ SD for duplicate analyses on two separate extractions.

[c] $\bar{X} \pm$ SD for foods obtained from K and R.

Table 13 -- Percent relative difference of B_6 vitamer content in foods calculated by external standardization from values calculated by internal standardization

Food	Vendor	PAM	PAL	POL	Total B_6
		Relative difference (%)[a]			
Bread	K	0.0	8.0	24.4	19.8
	R	0.0	13.6	29.2	23.3
	Average	0.0	10.6	26.1	21.2
Milk	K	-8.0	-13.1	6.5	0.5
	R	10.6	5.1	17.5	12.7
	Average	3.0	-1.7	22.2	7.3
Peas	K	0.0	-9.8	8.2	3.8
	R	0.0	14.8	29.9	26.1
	Average	33.3	4.5	20.5	16.5

[a] Values are computed from $\frac{\bar{X}_{ext} - \bar{X}_{int}}{\bar{X}_{ext}} \times 100$ where $\bar{X}_{ext}$ and $\bar{X}_{int}$ are the corresponding mean values of B_6 vitamer content in foods calculated by external standardization and internal standardization respectively. Values are obtained from tables 10 and 12.

technique. Although DPOL possesses structural similarity to the B_6 vitamers, it is not found naturally in foods making this vitamin antagonist ideal for use as an internal standard for vitamin B_6 quantitation in foods. Additionally, DPOL meets the requirements for an internal standard since it was completely resolved from the other 3 B_6 compounds while at the same time eluting near the peaks of interest. The current researcher believes that either internal or external standardization could be used for analysis of B_6 vitamers in foods.

E. Recovery of B_6 vitamers added to foods

The percent recoveries of B_6 vitamers spiked at the beginning of the analysis (see Fig. 8) and just before injection are shown in Table 14. Individual values for Table 14 are found in Appendix 6. The average percent recovery of spiked PAL and POL in bread and milk is low -- ranging from 0.6 to 15.0% for PAL and from 3.7 to 66.5% for POL. The recovery values for these 2 vitamers in the 2 foods were not consistent. On the whole percent recoveries of the vitamers spiked just before injection were consistent and close to 100% (mean recovery value = 92.4%).

Several authors have reported that natural vitamin B_6 is largely bound to food protein (51, 71, 53). The binding of vitamin B_6 in the form of ϵ-pyridoxyllysine has also been reported (51, 52). It was speculated that the low recovery values for some of the B_6 vitamers could be due to binding of the B_6 vitamers to a protein moiety in the foods. Using information from Tables 8 and 14 the coefficient of

Table 14 -- % recovery of B_6 vitamers in selected foods by HPLC

Food	Vendor	Spiked at beginning			Spiked before injection		
		PAM	PAL	POL	PAM	PAL	POL
Bread	K	90.4[a] ±9.4	-4.2[a] ±12.7	11.4[a] ±6.1	111.3[a] ±3.4	78.9[a] ±9.2	71.3[a] ±12.8
	R	79.0 ±16.1	15.0 ±36.3	66.5 ±1.3	111.3 ±21.5	60.4 ±7.1	79.5 ±19.0
	Average[b]	84.7 ±12.6	5.4 ±24.8	38.9 ±31.9	111.3 ±12.6	69.6 ±12.6	75.4 ±14.1
Milk	K	66.6 ±22.8	0.6 ±5.9	3.7 ±5.2	132.3 ±3.1	115.2 ±7.0	111.3 ±3.3
	R	69.0 ±18.2	5.4 ±7.6	10.1 ±9.0	73.3 ±5.2	53.3 ±5.0	97.7 ±0.6
	Average[b]	67.8 ±16.9	3.0 ±6.2	6.9 ±7.0	102.8 ±34.3	84.2 ±36.1	104.5 ±8.1
Peas	K	76.6 ±23.5	53.3 ±24.4	95.5 ±0.8	108.4 ±4.9	107.3 ±1.8	94.4 ±3.2
	R	78.0 ±26.9	69.5 ±3.7	98.5 ±51.5	79.3 ±17.0	83.6 ±18.7	95.4 ±1.8
	Average[b]	77.3 ±20.7	61.3 ±17.1	97.0 ±30.0	93.8 ±19.7	95.4 ±17.4	94.9 ±2.2

[a] $\bar{X} \pm$ SD for duplicate analyses on two separate extractions.

[b] $\bar{X} \pm$ SD for foods obtained from K and R.

Data calculated by external standardization

correlation (r) was calculated for the percent recovery of each spiked vitamer with respect to the crude protein content of the food to which B_6 spikes were added (Table 15). Negative correlations ranging from -0.62 to -0.78 were computed for all 3 vitamers. However the r values for all the 3 vitamers were all not significant at a significance level of 10% ($P \leq 0.1$). The difficulty encountered in liberating the B_6 vitamers quantitatively from the food in the extraction procedure (57) as well as some variation in the baseline values of the naturally occuring B_6 vitamers may well contribute to the overall inconsistency and low values in the percent recovery of some of the spiked vitamers. In contrast to the low percent recoveries of the spiked vitamers, the percent recovery of DPOL added at the same time as the B_6 vitamers to the food slurry was satisfactory. The recovery of DPOL added to bread, milk, and peas was 75%, 88% and 75%, respectively.

GC-EC analyses of B_6 vitamers in foods

A GC equipped with an EC detector was used for the analyses of B_6 vitamers in selected foods. The derivatizing reagent MBTFA was utilized to trifluoroacylate the B_6 vitamers.

A. External standardization

A typical chromatogram of B_6 standards separated by GC-EC is shown in Fig. 18. The separation of all 4 B_6 compounds was completed in less than 8 min. The order in which the vitamers were eluted was the reverse of the elution order encountered in the HPLC separation with

Table 15 -- Coefficient of correlation between percent recovery of spiked vitamers in foods and percent crude protein of the foods

B_6 vitamers	Coefficient of correlation, r	100 r^2 (%)[a]
PAM	-0.75 [b]	56.4
PAL	-0.62 [c]	38.4
POL	-0.78 [d]	60.8

[a] Proportion of the total variation of % recovery of spiked vitamers in a food attributed to the possible relationship with the % of crude protein in the food.

[b] r not significant at $P \leq 0.1$

[c] r not significant at $P \leq 0.1$

[d] r not significant at $P \leq 0.1$

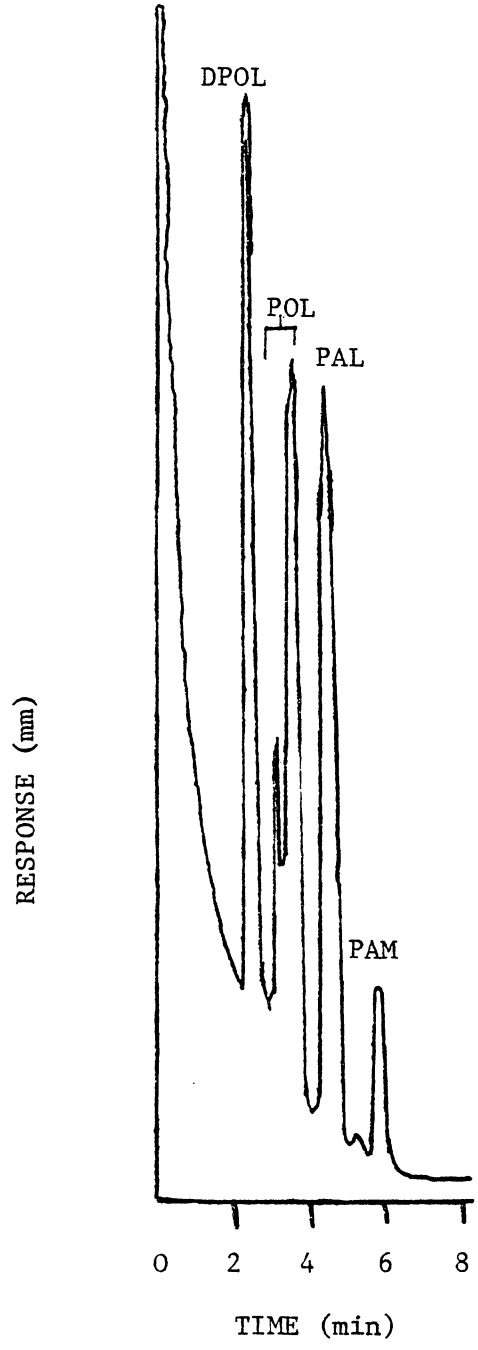


Fig. 18 -- Separation of B_6 standards by GC-EC

DPOL eluting first, followed by POL, PAL, and PAM. Trifluoroacylation of DPOL, PAL, and PAM gave rise to single peaks; whereas, trifluoroacylation of POL gave rise to 2 peaks: a major peak with a retention time of about 3.8 min and a minor peak with a retention time of approximately 3.2 min which sometimes appear as a shoulder to the major peak.

Korytnyk (38) had reported that in general trimethylsilylation of POL gave rise to 2 peaks with different retention times. The variation in sizes of the 2 peaks reported was dependent on the time the vitamer was exposed to the trimethylsilylation mixture. In this study, variation in sizes of the 2 peaks resulting from formation of the MBTFA derivative of POL was observed. The peak with the longer retention time always occurred as the predominant peak in repeated runs. In plotting the calibration curve of POL, the sum of the peak heights of both major and minor peaks were used. This method was found to give a satisfactory linear plot, indicating a probable quantitative reaction in the formation of 2 MBTFA derivatives of POL resulting in double peaks when chromatographed. The EC detector employs radioactive decay which involves exponential dependences of the measured signal on the concentration (72). The calibration curve using external standardization was thus plotted in logarithmic coordinates (Fig. 19) to yield a calibration curve which is expressed by the equation of a straight line:

$$\text{Log signal} = L\ (\log \text{concentration}) + \log \text{sensitivity}$$

where the linearity, L, is the proportionality constant in the

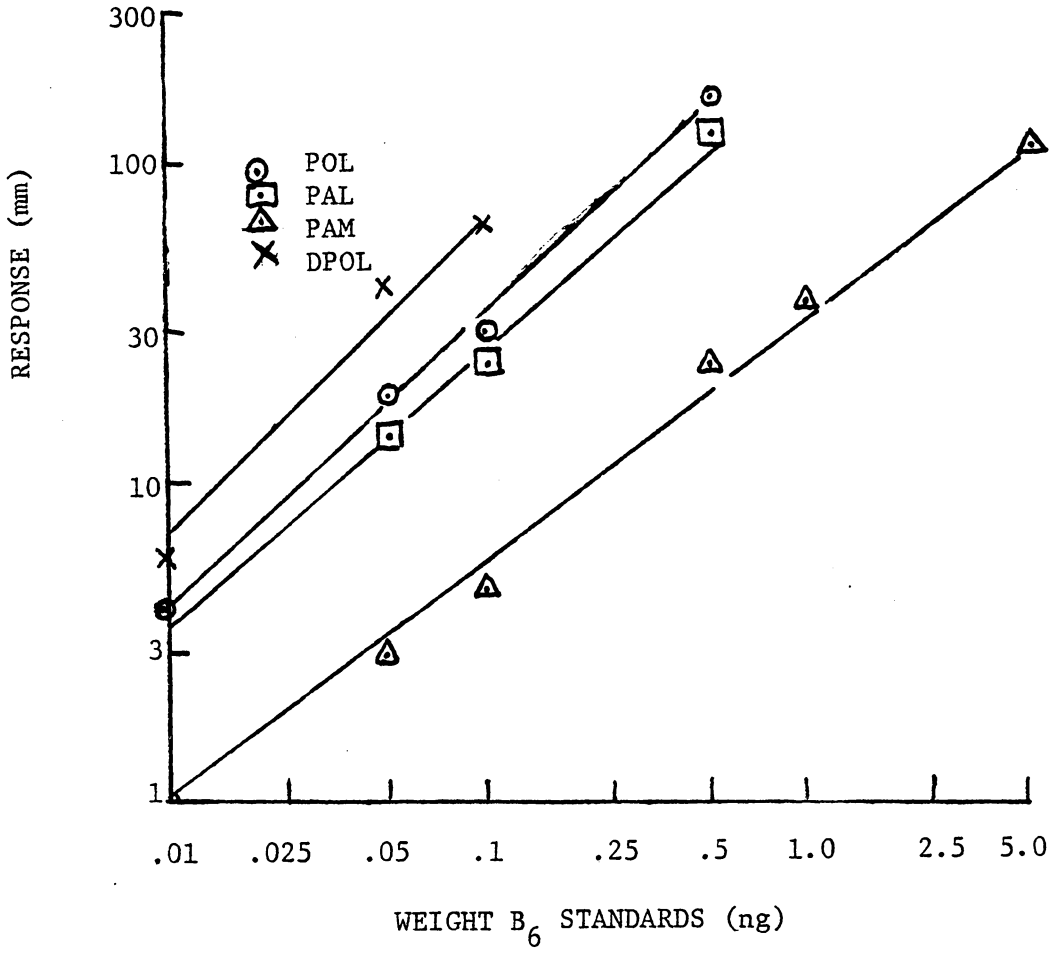


Fig. 19-- Representative curves of B_6 standards measured by GC-EC . Values were plotted on log-log graph paper.

relationship between the logarithm of the signal and the logarithm of the eluted B_6 compound and where L is the slope of the calibration curve. An intercept on the y-axis is equal to the logarithm of the sensitivity. Using information from Fig. 19, the relative sensitivities of the EC detector for the B_6 compounds were as follows: DPOL, 10^6; POL, 10^3; PAL, $2x10^3$; and PAM, 10^1. Also the linear range for DPOL was from 0.01 to 0.1 ng; for POL and PAL, from 0.01 to 0.5 ng; and for PAM, from 0.01 to approximately 5.0 ng. The lowest detectable amount for PAM, the limiting B_6 form in the chromatogram, was 0.01 ng at which concentration the peak height measurement was practical.

As is evidenced from Fig. 18 and Fig. 19, the amount of DPOL added at the beginning of the analysis to permit a reasonable DPOL peak in the HPLC analysis resulted in a DPOL peak which was beyond the EC detector's linearity. Dilution of the samples in order to reduce the DPOL peak size in the GC-EC chromatogram resulted in the loss of peaks corresponding to the B_6 vitamers. Unfortunately in this experiment several different concentrations of DPOL were not tried at the beginning of the spike. The concentration of the DPOL used gave a peak which was too large and the peak ht was beyond the linearity range of the EC detector for calculation purposes. The B_6 vitamer content in foods was thus calculated using only the external standardization method.

Several factors such as purity and dryness of the carrier gas, sensitivity of the capture process to detector temperature changes, column bleed (loss of liquid phase that coats the support within the column), column conditioning times, and electrical noises all contributed

to variability in the signal output in GC-EC techniques.

B. GC-EC analyses of food extracts

Chromatographic patterns of separated B_6 compounds in derivatized extracts of bread, milk, and peas are shown in Figure 20-22. In general, good separations with some interferences were obtained. There were slightly more interferences in the GC-EC separations than in the HPLC separations (Fig. 15-17). This may be due to the superior sensitivity of the EC detector which registered interferences not detected by the UV detector in HPLC. Interfering substances can also be introduced in the treatment steps, derivatization procedure, and dilution steps. Additionally, the fluctuating EC signal due to the inconstancy of experimental parameters also contributed to a variable baseline which may have made identification of small vitamer peaks difficult.

Location and identification of the B_6 vitamers were accomplished by matching peak patterns and retention times of the B_6 vitamers from foods with peak patterns of pure standards chromatographed in the same run. Further confirmation of the identity of each peak was also attempted by spiking with MBTFA derivative of the pure vitamer and by measuring the change in height of the original peak under study. GC-MS analysis was also used for final confirmation of the identity of each peak. However, the results for the GC-MS analysis were inconclusive. The problem encountered here in attempting to identify the peaks using GC-MS was not unlike that encountered in the attempt to identify the vitamer peaks in the HPLC separations. Here the amount of B_6 compounds

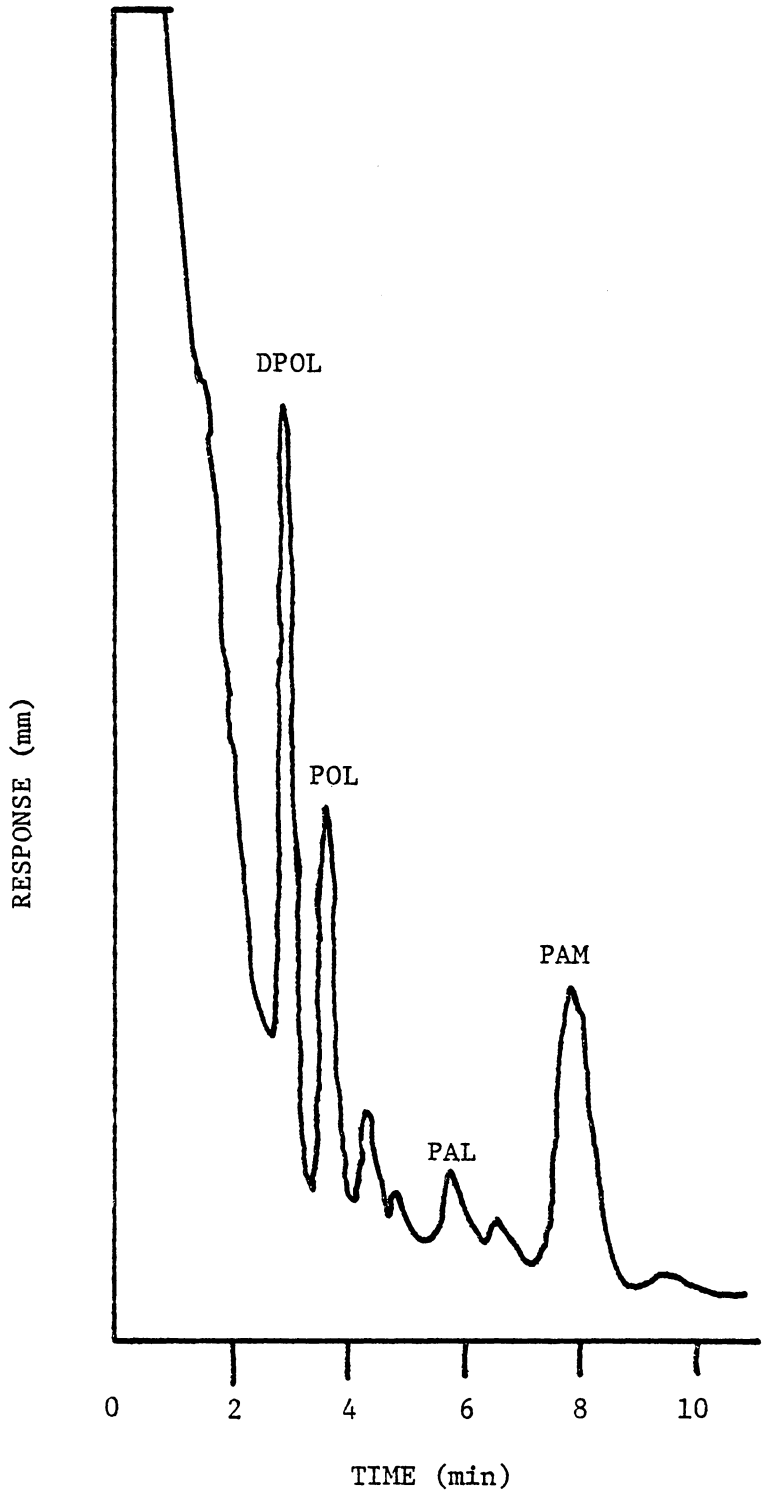


Fig. 20-- Separation of B_6 forms in bread by GC-EC

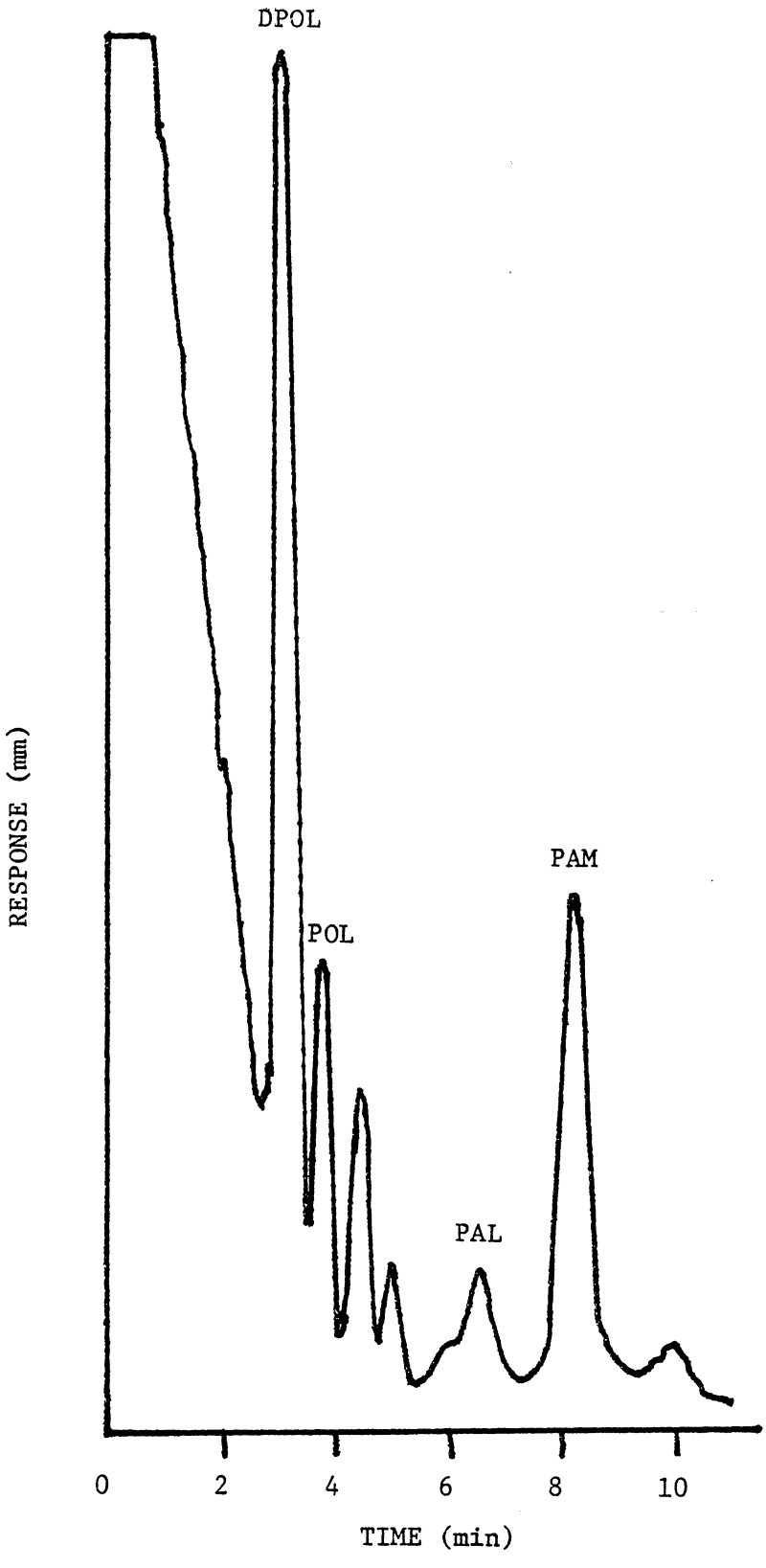


Fig. 21-- Separation of B_6 forms in milk by GC-EC

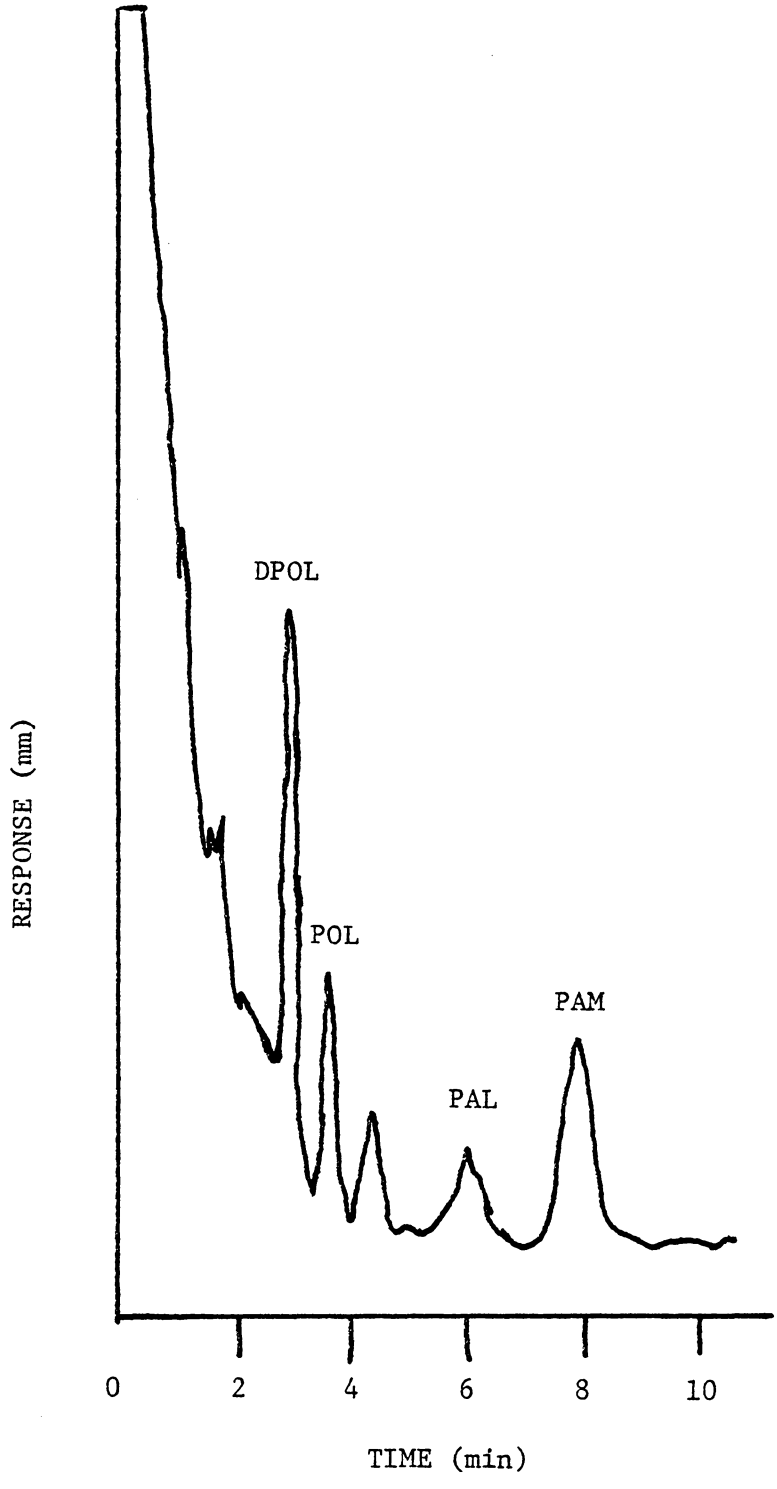


Fig. 22 -- Separation of B_6 forms in peas by GC-EC

sufficient for detection by EC (ng to pg range) was insufficient for detection by MS which has a sensitivity in the µg range. Further study is needed in resolving the problem of confirming the identity of peaks separated by GC-EC using the auxiliary technique of mass-spectrometry.

C. Calculation of B_6 vitamers in foods

Referring to the calibration curve in Fig. 19 for each vitamer, the mean response factor (MRF) of that vitamer equaled the slope of its calibration curve (y mm/ x mm). The vitamer peak height in the chromatogram of the food extract was then divided by the mean response factor (MRF) to give a x-coordinate in mm which corresponded to an equivalent weight of the vitamer in ng on the x-axis of the calibration curve. This value was the weight of the vitamer present in a known volume of sample injected into the GC. The amount of vitamer in 100 g of wet wt of food was then computed from the following formula:

$$\frac{\text{µg vitamer}}{\text{100 g wet wt food}} = \frac{b \times D \times 10^5}{y \times N}$$

Where y = volume of sample injected in µℓ

b = wt of vitamer in y µℓ injected sample as determined from calibration curve

D = serial dilution factor (dilution of MBTFA derivative with ethyl acetate prior to injection)

10^5 = dilution factor in treatment process prior to derivatization

N = wet wt of solids in g of a given volume of food slurry

Table 16 shows the mean values and standard deviations for B_6 vitamer content in bread, milk and peas as detected by GC-EC. The individual values for B_6 vitamer content in the same foods are shown in Appendix 7. There are no published values for GC analyses of B_6 vitamer contents of the foods analyzed in this study for comparison purposes. The B_6 vitamer content probably was affected by shelf-life.

Microbiological analyses of B_6 vitamers in foods

The AOAC method (61) with minor modifications was used for the classical microbiological assay for B_6 vitamer contents in the foods.

A. Calibration curves

Typical calibration curves of chromatographed and unchromatographed B_6 standards as determined by the S. uvarum assay are shown in Figures 10 and 11 respectively. The variation in the slope of the curve reflected the difference in the bioavailability of each vitamer with respect to S. uvarum. The degree of bioavailability of a vitamer is proportional to the slope of the calibration curve of the vitamer. Under the experimental conditions of this study, the bioavailability of the 3 vitamers with respect to S. uvarum were in decreasing order: POL, PAM, and PAL (Fig. 10 and 11). The linear range for all 3 vitamers was 5 ng beyond which the response curve became curvilinear. Using information from Fig. 10 and 11 a correction factor, L, which accounted for the loss of each vitamer from acid hydrolysis with autoclaving and ion-exchange chromatography was calculated as follows:

Table 16 -- GC-EC assay of B_6 vitamers in selected foods using external standardization[a]

Food	Vendor	PAM	PAL	POL	Total B_6
		(µg/100g wet wt)			
Bread	K	406.5[b] ±105.3	339.0[b] ±68.4	1041.2[b] ±239.8	1786.8[b] ±413.5
	R	268.9 ±39.4	79.1 ±35.4	875.6 ±390.9	1273.5 ±465.6
	Average[c]	337.8 ±102.6	234.0 ±129.1	958.4 ±281.5	1530.2 ±465.9
Milk	K	952.0 ±76.1	410.0 ±197.0	4577.7 ±638.3	5939.8 ±911.3
	R	1664.1 ±482.1	163.5 ±15.4	6117.5 ±550.3	7945.2 ±83.6
	Average[c]	1308.1 ±498.4	286.8 ±182.4	5347.6 ±1013.4	6942.5 ±1272.6
Peas	K	991.3 ±319.7	393.1 ±3.7	5325.9 ±1321.7	6710.3 ±998.3
	R	1356.0 ±179.8	158.7 ±27.9	8380.4 ±399.5	9895.0 ±551.4
	Average[c]	1173.6 ±298.6	275.9 ±136.3	6853.1 ±1935.3	8302.7 ±1953.0

[a] Values not corrected for % recovery.

[b] $\bar{X} \pm$ SD for duplicate analyses on two separate extractions.

[c] $\bar{X} \pm$ SD for foods obtained from K and R.

$$L = \frac{100}{100 - 100\,\frac{S_1 - S_2}{S_1}}$$

Where S_1 = slope of the calibration curve of unchromatographed vitamer

S_2 = slope of the calibration curve of chromatographed vitamer

B. Calculation of B_6 vitamer content in foods

The percent loss due to the extraction procedure (from acid hydrolysis with autoclaving) and column chromatography was calculated as, 18.8% for PAM, 6.8% for PAL, and 6.29% for POL. The B_6 vitamer content in a food was calculated as follows:

$$\mu g \text{ vitamer/100 g wet wt food} = \frac{x}{y} \times (12500) \times (0.05) \times F \times L$$

Where y = volume of extract inoculated with S. uvarum

x = wt of vitamer in ng in y ml of inoculated extract obtained from the calibration curve of chromatographed B_6 vitamer standard

(12500)= factor for serial dilutions of extract and for expressing the wt of vitamer in ng per 2 g freeze dried wt of food

(0.05) = conversion factor to express the vitamer content as µg vitamer/100 g freeze-dried wt of food

F = conversion factor to express vitamer content as µg vitamer/100 g wet wt of food

L = correction factor for loss due to acid hydrolysis with autoclaving and column chromatography of vitamers as determined from the difference in the slope of calibration curves of the chromatographed and unchromatographed standard.

The calculated B_6 vitamer contents in bread, milk, and peas are shown in Table 17 (mean and standard deviation values) and Appendix 8 (individual values). Variations in vitamin B_6 values among laboratories for selected foods have been reported (4, 73, 74). Toepfer and Polansky (10) reported the overall percent coefficient of variations for B_6 contents in foods determined by 9 collaborators as follows: total vitamin B_6, 18.9%; POL, 22.9%, PAL, 24.8%; and PAM, 27.8%. Recognizing the possibility of variation in reported values, the B_6 vitamer values in the 3 foods obtained in this study were found in general to agree closely with reported B_6 vitamer values determined by the S. uvarum method for similar foods (Table 18). Recovery data were not obtained.

Comparison of data obtained by HPLC, GC-EC, and microbiological analyses

A. Values for vitamin B_6 content by 3 methods of analyses

There were in general 3 categories of data for the B_6 vitamer content

Table 17 -- Microbiological assay of B_6 vitamers in selected foods[a]

Food	Vendor	PAM (µg/100g wet wt)	PAL (µg/100g wet wt)	POL (µg/100g wet wt)	Total B_6 (µg/100g wet wt)
Bread	K	51.5[a] ±10.1	84.1[a] ±8.1	30.1[a] ±3.0	165.7[a] ±15.6
	R	44.0 ±9.0	56.9 ±4.2	17.7 ±1.6	118.7 ±7.4
	Average[b]	48.3 ±9.7	72.5 ±15.8	23.9 ±7.1	142.2 ±27.5
Milk	K	126.3 ±14.8	391.9 ±105.8	85.3 ±9.8	603.5 ±118.2
	R	79.9 ±9.5	361.8 ±67.0	66.4 ±7.9	508.2 ±52.8
	Average[b]	103.1 ±27.3	375.2 ±81.8	74.8 ±12.9	555.9 ±98.9
Peas	K	13.7 ±7.2	2.4 ±0.2	13.4 ±3.3	30.6 ±2.8
	R	19.7 ±2.8	3.5 ±0.7	17.8 ±2.0	40.3 ±2.7
	Average[b]	16.7 3.7	3.0 ±0.8	15.5 ±3.4	35.4 ±5.8

[a] $\bar{X} \pm$ SD of quadruplicate analyses

[b] $\bar{X} \pm$ SD for foods obtained from K and R

Table 18 -- Percent distribution of B_6 vitamers in selected foods as determined by HPLC, GC-EC and S. uvarum methods

Food	Assay	PAM	PAL	POL
		% of Total B_6[a]		
Bread	HPLC	0.0	36.1	63.9*
	GC-EC	22.2	15.0	62.7*
	S. uvarum	35.7	50.0*	14.3
	S. uvarum[b]	30.2	37.2*	32.6
Milk	HPLC	13.3	33.3	66.7*
	GC-EC	18.9	4.2	77.1*
	S. uvarum	16.7	66.7*	11.7
	S. uvarum[c]	23.7	73.7*	2.6
Peas	HPLC	0.0	28.7	71.2*
	GC-EC	14.1	3.2	82.5*
	S. uvarum	48.6*	8.6	42.8
	S. uvarum[d]	38.7*	33.7	28.7

* Predominant B_6 vitamer in sample.

[a] Individual B_6 vitamer value and Total B_6 value obtained from Table 19.

[b] Polansky et al., 1964; enriched white bread.

[c] Orr, 1969; non-fat dried milk.

[d] Polansky, 1969; frozen sweet green peas.

in the foods as determined by GC-EC (table 19). The first category are those GC-EC values which are larger than the corresponding HPLC values. This category of values occurred for PAM in all 3 foods analyzed. In the second category are those GC-EC values which are smaller than the corresponding HPLC values suggesting the possibility of incomplete derivatization. The last category includes those GC-EC values which are in reasonable agreement with the HPLC values. These various categories could be caused by the following parameters: (1) interfering compounds eluting with the particular vitamer, (2) incomplete recovery of vitamers during extraction, (3) incomplete derivatization in the GC-EC method.

Determination of percent recoveries of the standards from the derivatization process in GC-EC itself showed that approximately 89.4% of PAM, 71.2% of PAL, and 65.1% of POL were recovered (Appendix 9). Despite satisfactory recovery rate of the 3 vitamer standards, further study is needed on the quantitative mechanisms of the derivatization process and interaction of the derivatizing reagent with naturally occurring B_6 vitamers in food extracts. Additionally further work is needed to determine the possibility of contamination which may lead to interfering or artifactual peaks which are amplified by the high sensitivity of the GC-EC thus further aggravating the problem.

On the whole the S. uvarum B_6 values of this study agree well with reported vitamin B_6 values of other S. uvarum analyses. In comparing the HPLC values of the 3 vitamers in the 3 foods to their corresponding

Table 19 -- Comparison of vitamin B_6 contents in foods as determined by HPLC, GC-EC and S. uvarum method to reported values for similar foods.

Food	Assay	PAM	PAL	POL	Total B_6
		mg/100g wet wt			
Bread	HPLC[a]	0.000	1.790	3.170	4.960
	GC-EC[a]	0.340[f]	0.230[g]	0.960[g]	1.530
	S. uvarum[a]	0.050	0.070	0.020	0.140
	S. uvarum[b]	0.013	0.016	0.014	0.043
Milk	HPLC[a]	0.080	0.200	0.400	0.600
	GC-EC[a]	1.310[f]	0.290[h]	5.350[f]	6.940
	S. uvarum[a]	0.100	0.400	0.070	0.600
	S. uvarum[c]	0.090	0.280	0.010	0.380
	S. uvarum[d]	-	-	-	0.350
Peas	HPLC[a]	0.000	2.300	5.700	8.000
	GC-EC[a]	1.170[f]	0.270[g]	6.850[h]	8.300
	S. uvarum[a]	0.017	0.003	0.015	0.035
	Fluorometry[c]	0.003	0.023	0.023	0.050
	S. uvarum[e]	0.031	0.027	0.023	0.080

[a] $\overline{X}$ for foods obtained from K and R.

[b] Polansky et al., 1964; enriched white bread.

[c] Orr, 1969.

[d] Total B_6 value calculated from information on box label of Carnation instant non-fat dry milk fortified with vitamins A and D.

[e] Polansky, 1969; frozen sweet green peas.

[f] GC-EC value > HPLC value possibly due to interfering material in GC-EC analysis.

[g] GC-EC value < HPLC value possibly due to incomplete derivatization in GC-EC analysis.

[h] GC-EC value ≃ HPLC value.

S. uvarum values, the values for PAM by both methods are so low that a good comparison cannot be made. PAL values by both methods are comparable for milk, being much higher as detected by HPLC in bread and peas. POL values in all 3 foods are considerably higher as detected by HPLC than their corresponding S. uvarum values. The overall B_6 vitamer values in all 3 foods as detected by GC-EC are considerably higher than the corresponding values detected by S. uvarum.

B. Percent distribution of the 3 vitamers as detected by HPLC, GC-EC, and S. uvarum

Table 18 shows the distribution of the B_6 vitamers as a percentage of the total B_6 content in the food. POL was the major vitameric form in all food samples as detected by HPLC and GC-EC methods. S. uvarum assay showed that PAL predominated in bread and milk and PAM in peas. That the predominant B_6 vitamers as detected by S. uvarum are different from those as detected by the physico-chemical methods (HPLC and GC-EC) suggested differences in the bioavailability of the 3 B_6 vitamers with respect to S. uvarum.

C. Percent coefficient of variation

The percent coefficient of variation of the total vitamin B_6 values as determined by S. uvarum assay -the classical method- ranged from 6.2 to 19.6% (see Appendix 10) and was comparable to the percent coefficient of variation for B_6 values of foods determined by this method as reported by other authors (3, 4, 6, 8, 10, 11). A general comparison of the percent coefficient of variation of B_6 values obtained by the 3 methods

indicated that the precision (reproducibility) of the HPLC method is comparable to that of the S. uvarum method, whereas the precision of the GC-EC method is much less than that of either the HPLC or S. uvarum methods. This suggests the need for further improvement of the precision of both the HPLC and GC-EC methods preferably to obtain a percent coefficient of variation of from 5 to 10%.

D. Magnitude and percent distribution of errors

Tables 20-22 show the magnitude and distribution of error in determining PAM, PAL, and POL respectively by HPLC. A summary of the individual error values is found in Table 23. From Table 23 it is observed that the instrument percent error is much smaller than the combined percent error due to instrument and treatment. For example the determination of POL in foods by HPLC is associated with an average percent instrumental error of 1.6% (range, 0 to 3.2%) as compared to a combined percent error due to instrument and treatment of 11.7% (range, 0.4 to 21.5%). This larger combined instrument and treatment value may be a reflection of the difficulty with the extraction procedure. Table 24 summarizes the magnitude and distribution of error in determining PAM, PAL, and POL by GC-EC (See tables 25-27 for the magnitude and distribution of error for individual vitamer). The percent instrument error for the GC-EC method was somewhat high approximating the combined percent error values for instrument and treatment in some cases. For PAM the percent instrument error was 10.8% (range, 0.9 to 30.4%) as compared to a percent error value due to instrument and treatment of

Table 20 -- Magnitude and distribution of error in determining PAM in foods by HPLC

Food	Vendor		Mean[a] Pk Ht (mm)	Pk Ht 1 (mm)	Pk Ht 2 (mm)	Inst.[b] Error (%)	Mean[c] PAM content (µg/100g wet wt)	Individual PAM content (µg/100g wet wt)	Inst. + trt.[d] error (%)
Bread	K	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0			
	R	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0			
Milk	K	1	2.5	*	2.5	*	69.0	76.6	11.0
		2	2.0	2.0	2.0	0.0			
	R	1	3.0	3.0	3.0	0.0	88.4	88.8	0.4
		2	3.0	*	3.0	*			
Peas	K	1	0.0	0.0	0.0	0.0	0.5	0.9	80.0
		2	2.2	2.0	2.5	11.1			
	R	1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
		2	0.0	0.0	0.0	0.0			

[a] Mean of duplicate Pk Ht values.

[b] Calculated from $\frac{\text{mean Pk Ht - Pk Ht 1}}{\text{mean Pk Ht}} \times 100$.

[c] mean of duplicate values of PAM content in food.

[d] Calculated from $\frac{\text{mean PAM content - Individual PAM content}}{\text{mean PAM content}} \times 100$.

* Data rejected because of error probably due to instrument and/or treatment.

**Value not calculated because of rejected data.

Table 21 -- Magnitude and distribution of error in determining PAL in foods by HPLC.

Food	Vendor		Mean[a] Pk Ht (mm)	Pk Ht 1 (mm)	Pk Ht 2 (mm)	Inst.[b] Error (%)	Mean[c] PAM content (µg/100g wet wt)	Individual PAM content (µg/100g wet wt)	Inst. + trt.[d] error (%)
Bread	K	1	55.5	54.0	57.0	2.7	1972.1	2292.2	16.2
		2	40.0	41.0	39.0	2.5			
	R	1	59.0	58.0	60.0	1.7	1601.6	1918.7	19.8
		2	39.5	39.0	40.0	1.3			
Milk	K	1	5.0	5.0	5.0	0.0	158.8	192.5	21.2
		2	3.2	3.0	3.5	7.7			
	R	1	5.7	5.5	6.0	4.3	223.0	213.7	4.2
		2	6.2	6.5	6.0	4.0			
Peas	K	1	42.0	42.0	42.0	0.0	2218.7	2179.8	1.7
		2	43.5	*	43.5	**			
	R	1	41.0	42.0	40.0	2.4	2360.0	2303.8	2.4
		2	43.0	43.0	43.0	0.0			

[a] Mean of duplicate Pk Ht values.

[b] Calculated from $\frac{\text{mean Pk Ht - Pk Ht 1}}{\text{mean Pk Ht}} \times 100$.

[c] Mean of duplicate values of PAM content in food.

[d] Calculated from $\frac{\text{mean PAM content - Individual PAM content}}{\text{mean PAM content}} \times 100$.

* Data rejected because of error probably due to instrument and/or treatment.

**Value not calculated because of rejected data.

Table 22 -- Magnitude and distribution of error in determining POL in foods by HPLC

Food	Vendor		Mean[a] Pk Ht (mm)	Pk Ht 1 (mm)	Pk Ht 2 (mm)	Inst.[b] Error (%)	Mean[c] PAM content (µg/100g wet wt)	Individual PAM content (µg/100g wet wt)	Inst. + trt.[d] error (%)
Bread	K	1	72.0	71.0	73.0	1.4	4188.3	4543.2	8.5
		2	60.7	61.0	60.5	0.4			
	R	1	50.7	49.5	52.0	2.5	2154.9	1788.5	17.0
		2	36.0	37.0	35.0	2.8			
Milk	K	1	6.0	6.0	6.0	0.0	382.3	352.9	7.7
		2	7.0	7.0	7.0	0.0			
	R	1	7.7	7.5	8.0	3.2	362.0	440.0	21.5
		2	5.0	5.0	*	**			
Peas	K	1	80.5	81.0	80.0	0.6	5728.7	6382.8	11.4
		2	64.0	66.0	62.0	3.1			
	R	1	66.0	66.0	*	**	5686.2	5664.8	0.4
		2	66.5	68.0	65.0	2.2			

[a] Mean of duplicate Pk Ht values.

[b] Calculated from $\frac{\text{mean Pk Ht - Pk Ht 1}}{\text{mean Pk Ht}} \times 100$.

[c] Mean of duplicate values of PAM content in food.

[d] Calculated from $\frac{\text{mean PAM content - Individual PAM content}}{\text{mean PAM content}} \times 100$.

* Data rejected because of error probably due to instrument and/or treatment.

**Value not calculated because of rejected data.

Table 23 -- Magnitude and distribution of error in determining B_6 vitamers in foods by HPLC

Food	Source	Extraction No.	PAM Inst. Error (%)	PAM Inst. + trt. Error (%)	PAL Inst. Error (%)	PAL Inst. + trt. Error (%)	POL Inst. Error (%)	POL Inst. + trt. Error (%)
Bread	K	1	0.0	0.0	2.7	16.2	1.4	8.5
		2	0.0		2.5		0.4	
	R	1	0.0	0.0	1.7	19.8	2.5	17.0
		2	0.0		1.3		2.8	
Milk	K	1	**	11.0	0.0	21.2	0.0	7.7
		2	0.0		7.7		0.0	
	R	1	0.0	0.4	4.3	4.2	3.2	21.5
		2	**		4.0		**	
Peas	K	1	0.0	80.0	0.0	1.7	0.62	11.4
		2	11.1		**		3.1	
	R	1	0.0	0.0	2.4	2.4	**	0.4
		2	0.0		0.0		2.2	
Average			1.1 ± 3.5	15.2 ± 32.0	2.4 ± 2.3	10.9 ± 9.1	1.6 ± 1.3	11.1 ± 7.4
Range			(0-11.1)	(0-80)	(0-4.3)	(2.4-21.2)	(0-3.2)	(0.4-21.5)

** Value not calculated because of rejected data.

14.5% (range 5.6 to 22.8%). This higher percent error distribution due to instrument may be due in part to the inconstancy of EC signal output. Despite the control of operational parameters, variations in comparative detector responses published in the literature have been reported (72). The author believes that the HPLC values are more reliable because of better reproducibility of data.

E. Extraction difficulty as a possible factor contributing to variations in data

The larger percent error due to instrument and treatment in both HPLC and GC-EC may well be a reflection of the inconstancy and/or inefficiency of the extraction procedure. Vanderslice et al. (57) have developed a simple and excellent extraction procedure using sulfosalicylic acid as a deproteinating agent for the recovery of all forms of B_6 from foods; this procedure was found to yield clean samples from quantitative analysis by column chromatography. The chromatographic system used by Vanderslice et al. is somewhat complicated involving several components including dual resin columns, and fluorescence spectrophotometry.
It is suggested that future work include investigating the possibility of combining the simple extraction procedure using sulfosalicylic acid with the HPLC system reported in this study which the researcher feels to be a much simplier, efficient, and a more practical method than that of Vanderslice and co-workers.

Table 24 -- Magnitude and distribution of error in determining B_6 vitamers in foods by GC-EC

Food	Source	Extraction No.	PAM		PAL		POL	
			Inst. Error (%)	Inst. + trt. Error (%)	Inst. Error (%)	Inst. + trt. Error (%)	Inst. Error (%)	Inst. + trt. Error (%)
Bread	K	1	16.5	18.3	17.9	14.3	9.7	16.3
		2	**		28.6		4.8	
	R	1	2.8	10.4	3.7	31.6	4.5	31.6
		2	0.9		21.4		11.1	
Milk	K	1	15.4	5.6	**	34.0	7.7	9.9
		2	19.6		28.6		**	
	R	1	12.9	20.5	17.9	6.7	5.3	9.9
		2	30.4		2.1		**	
Peas	K	1	9.1	22.8	26.5	0.7	22.2	17.5
		2	2.7		18.7		1.0	
	R	1	4.2	9.4	28.7	12.4	**	3.4
		2	4.8		8.5		19.6	
	Average		10.8 ± 9.1	14.5 ± 6.9	18.4 ± 9.8	16.6 ± 13.4	9.5 ± 7.1	14.8 ± 9.7
	Range		(0.9-30.4)	(5.6-22.8)	(2.1-28.7)	(0.7-34.0)	(1.0-22.2)	(3.4-31.6)

** Value not calculated because of rejected data.

Table 25 -- Magnitude and distribution of error in determining PAM in foods by GC-EC.

Food	Vendor		Mean[a] PAM content (µg/100g wet wt)	Individual PAM content 1 (µg/100g wet wt)	Individual PAM content 2 (µg/100g wet wt)	Inst.[b] Error (%)	Mean[c] PAM content (µg/100g wet wt)	Individual PAM content (µg/100g wet wt)	Inst. + trt.[d] error (%)
Bread	K	1	481.0	401.4	560.6	16.5	406.5	481.0	18.3
		2	332.2	*	332.2	**			
	R	1	241.0	234.2	247.8	2.8	268.9	241.0	10.4
		2	296.8	299.5	294.1	0.9			
Milk	K	1	1005.8	851.1	1160.5	15.4	952.0	1005.8	5.6
		2	898.2	722.1	1074.3	19.6			
	R	1	1323.2	1151.9	1494.4	12.9	1664.1	1323.2	20.5
		2	2005.0	2615.2	1394.8	30.4			
Peas	K	1	765.2	695.6	834.8	9.1	991.3	765.2	22.8
		2	1217.4	1182.6	1252.2	2.7			
	R	1	1228.8	1177.0	1280.6	4.2	1356.0	1228.8	9.4
		2	1483.1	1412.4	1553.7	4.8			

[a] Mean of duplicate values of PAM content at instrument level.

[b] Calculated from $\frac{\text{mean PAM content - Individual PAM content 1}}{\text{mean PAM content}} \times 100$.

[c] Mean of duplicate values of PAM content in food at instrument and treatment level.

[d] Calculated from $\frac{\text{mean PAM content - Individual PAM content}}{\text{mean PAM content}} \times 100$.

* Data rejected because of error probably due to instrument and/or treatment.

**Value not calculated because of rejected data.

Table 26 -- Magnitude and distribution of error in determining PAL in foods by GC-EC

Food	Vendor		Mean[a] PAM content (µg/100g wet wt)	Individual PAL content 1 (µg/100g wet wt)	Individual PAL content 2 (µg/100g wet wt)	Inst.[b] Error (%)	Mean[c] PAM content (µg/100g wet wt)	Individual PAM content (µg/100g wet wt)	Inst. + trt.[d] error (%)
Bread	K	1	387.4	318.2	456.6	17.9	339.0	387.4	14.3
		2	290.6	207.5	373.6	28.6			
	R	1	104.1	107.9	100.3	3.7	79.1	104.1	31.6
		2	154.0	121.1	186.8	21.4			
Milk	K	1	549.3	549.3	*	**	410.0	549.3	34.0
		2	270.8	193.4	348.2	28.6			
	R	1	174.4	143.2	205.5	17.9	163.5	174.4	6.7
		2	152.6	149.4	155.7	2.1			
Peas	K	1	390.5	287.0	493.9	26.5	393.1	390.5	0.7
		2	395.7	321.7	469.6	18.7			
	R	1	178.4	127.1	229.7	28.7	158.7	178.4	12.4
		2	138.9	127.1	150.6	8.5			

[a] Mean of duplicate values of PAM content at instrument level.

[b] Calculated from $\frac{\text{mean PAM content - Individual PAM content 1}}{\text{mean PAM content}} \times 100$.

[c] Mean duplicate values of PAM content in food at instrument and treatment level.

[d] Calculated from $\frac{\text{mean PAM content - Individual PAM content}}{\text{mean PAM content}} \times 100$.

* Data rejected because of error probably due to instrument and/or treatment.

**Value not calculated because of rejected data.

Table 27 -- Magnitude and distribution of error in determining POL in foods by GC-EC.

Food	Vendor		Mean[a] PAM content (µg/100g wet wt)	Individual POL content 1 (µg/100g wet wt)	Individual POL content 2 (µg/100g wet wt)	Inst.[b] Error (%)	Mean[c] PAM content (µg/100g wet wt)	Individual PAM content (µg/100g wet wt)	Inst. + trt.[d] error (%)
Bread	K	1	1210.8	1093.1	1328.4	9.7	1041.2	1210.8	16.3
		2	871.6	913.2	830.0	4.8			
	R	1	599.2	571.9	626.4	4.5	875.6	599.2	31.6
		2	1152.0	1280.0	1024.0	11.1			
Milk	K	1	5029.1	4642.2	5415.9	7.7	4577.7	5029.1	9.9
		2	4126.4	4126.4	*	**			
	R	1	6506.7	6849.3	6164.0	5.3	6117.5	6506.7	9.9
		2	5728.4	5728.4	*	**			
Peas	K	1	6260.5	4869.0	7652.0	22.2	5325.9	6260.5	17.5
		2	4391.3	4434.8	4347.8	1.0			
	R	1	8097.9	8097.9	*	**	8380.4	8097.9	3.4
		2	8662.9	10357.8	6968.0	19.6			

[a] Mean of duplicate values of PAM content at instrument level.

[b] Calculated from $\frac{\text{mean PAM content - Individual PAM content 1}}{\text{mean PAM content}} \times 100$.

[c] Mean of duplicate values of PAM content in food at instrument and treatment level.

[d] Calculated from $\frac{\text{mean PAM content - Individual PAM content}}{\text{mean PAM content}} \times 100$.

* Data rejected because of error probably due to instrument and/or treatment.

**Value not calculated because of rejected data.

CONCLUSIONS

In this study 2 physico-chemical methods -- HPLC and GC-EC -- have been developed and explored as alternative methods to the classical microbiological method using S. uvarum for the separation and quantitation of POL, PAL, and PAM in selected foods. All 3 vitamers POL, PAL, and PAM were separated and quantitated by all 3 methods.

Successful separation and quantitation of pure vitamin B_6 compounds POL, PAL, PAM, and DPOL were obtained by the HPLC method. The HPLC technique presented the following advantages; good sensitivity, large range in linearity of the UV detector response, simplicity of method, instrumentation, non-destructive absorbance detector, short analysis time, and good precision.

In this study a method for the detection and quantitation of the MBTFA derivatives of pure forms of POL, PAL, PAM, and DPOL using an EC detector was also developed. The short separation time and exceptional sensitivity of the EC detector suggested potential use of the GC-EC system for the detection and quantitation of B_6 vitamers in foods.

In general the HPLC and GC-EC methods yielded higher values for B_6 vitamer contents in foods than the microbiological method.

The precision of the HPLC and especially the GC-EC method could be improved; both of the chromatographic methods show promise for use in food analyses. Spiking and retention times were used to identify the vitamer peaks in both chromatographic methods; MS confirmation would be desirable. Better extraction and derivatization procedures would also be appropriate.

Gregory (53) has reported microbiological and HPLC values for total vitamin B_6 in breakfast cereals which were comparable to each other. However, Vanderslice (57) and the current researcher found that the HPLC values were much greater than the microbiological values for total vitamin B_6 in the foods which each researcher evaluated. Although there are some problems associated with the HPLC and GC-EC methods at this stage, both methods do offer potential solutions for a better and more reliable quantitation of B_6 vitamers in foods which will then permit studies on vitamin B_6 requirements in humans.

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Appendix 1 -- Preparation of culture media for Saccharomyces uvarum assay for B_6 vitamers in foods

Precautions: All solutions containing vitamin B_6 were protected from light by working in a darken lab and covering all receptacles containing vitamin B_6 in aluminium foil.

A. Liquid culture medium

1. Standard solutions of B_6 vitamers

a. Stock solutions (10 µg/ml) of each B_6 vitamers were prepared by dissolving 0.01216 g POL·HCl, 0.01218 g PAL·HCl and 0.01434 g PAM·2HCl respectively in 1.0 N HCl in 1 L volumetric flasks and diluting each flask to 1 L with 1.0 N HCl. The stock solutions were stored in 1 L dark glass bottles at $5^{o}C$ until use.

b. Intermediate solutions (1.0 µg/ml) were prepared by diluting 10 ml of each of the stock solution to 100 ml in water in 100 ml water in 100 ml volumetric flasks.

c. Working solutions (1.0 ng/ml) were prepared by diluting 1.0 ml of each of the intermediate solutions to 100 ml with water in 100 ml volumetric flasks. Ten milliliters of each of these prepared solutions were then further diluted to 100 ml

with water to give the working solutions of 1.0 ng/ml concentration of each B_6 vitamer.

2. Mixed solution of POL, PAL, and PAM

This was prepared by pipetting 20.0 ml of each intermediate solution prepared previously into a 1 L volumetric flask and diluting to volume with water.

3. Basal medium stock solution

Fifty-three grams of Bacto-Pyridoxine-Y-medium (Catalog No. 0951-15-2, Difco Labs, Detroit, MI) were dissolved in 1 L water in a 1 L Erlenmeyer. The mixture was steamed for 10 min at 100°C. The mixture was stored in a pyrex bottle with screw cap at 5°C until use (the mixture was prepared less than 24 hr before use).

4. Liquid culture media

Five milliliters of the mixed solution of POL, PAL and PAM were pipetted into 10 16x150 mm disposable pyrex test-tubes containing 2 4 mm glass beads. The tubes were covered with Kim caps and autoclaved 10 min at 121°C. Five milliliters of steamed vitamin B_6 free basal medium (Bacto-Pyridoxine-Y-medium) was added under aseptic conditions. The prepared medium was

stored at 5°C until use.

B. Agar culture medium

Twenty five grams of Bacto-Y-M agar (Catalog No. 0712-01-8, Difco Labs, Detroit, MI) were suspended in 400 ml water in a 500 ml Erlenmeyer. The level of the solution was marked with a marking pencil.

The solution was heated over a hot plate with a magnetic stirrer - to prevent charring of the medium in the bottom of the beaker - until the agar dissolves. The volume was then adjusted back to 500 ml with hot(60°C) -- to prevent the agar from solidifying.

About 10 ml portions of the hot agar (kept at 60°C) were pipetted into 50 16x150 mm test-tubes. The tubes were covered with Kim caps and autoclaved 15 min at 121°C. The tubes with the hot agar were then tilted on a rubber hose (1/2 inch diameter Tygon tubing) to form slants and cooled in this position.

C. Inoculum rinse

Five milliliters of water was pipetted into 50 16x150 mm test-tubes. The tubes were covered with Kim caps and autoclaved 10 min at 121°C.

Five milliliters of steamed Bacto-Pyridoxine-Y-medium was added under aseptic condition with a pipet. The tubes were stored at $5^{o}C$ until use.

Appendix 2 -- Gram Staining

Materials

Slides with prepared smears

Gram staining kit and wash bottle

paper toweling

1. Cover the smear with crystal violet. Let stand for 20 sec
2. Briefly wash off the stain with wash bottle (not more than 2 or 3 sec).
3. Cover the smear with Gram's iodine for 60 sec.
4. Pour off the Gram's iodine and flood the smear with 95% ethyl alcohol for 15 to 20 sec.
5. Stop the action of the alcohol by rinsing the slide with wash bottle for a few sec.
6. Cover smear with safranin for 20 sec.
7. Wash gently for a few sec, blot with paper toweling and let dry at room temperature.
8. Examine slide under oil immersion lens immediately.

Appendix 3 -- Individual values for HPLC assay of B_6 vitamers in selected foods using external standardization[a]

Food	Vendor	PAM	PAL	POL	Total B_6
		(µg/100g wet wt)			
Bread	K	0.0	2292.2	4543.2	6835.4
		0.0	1652.0	3833.3	5485.3
	R	0.0	1918.7	2521.3	4440.0
		0.0	1284.5	1788.5	3073.0
Milk	K	76.6	192.5	352.9	622.0
		61.3	125.1	411.7	598.1
	R	88.8	213.7	440.0	742.5
		88.0	232.3	283.9	605.0
Peas	K	0.0	2179.8	6382.8	8562.6
		0.9	2257.6	5074.6	7332.2
	R	0.0	2303.8	5664.8	7968.6
		0.0	2416.2	5707.7	8123.9

[a] Values not corrected for % recovery.

Appendix 4 -- Individual values for HPLC assay of B_6 vitamers in selected foods using internal standardization[a]

Food	Vendor	PAM	PAL	POL	Total B_6
		(µg/100g wet wt)			
Bread	K	0.0	2490.3	6009.3	8499.6
		0.0	1799.0	5066.8	6865.8
	R	0.0	2082.5	3335.0	5417.5
		0.0	1626.6	2756.5	4383.1
Milk	K	71.7	172.1	383.0	626.8
		56.2	108.7	435.2	600.1
	R	77.8	175.8	439.9	693.5
		118.6	294.2	438.0	850.8
Peas	K	0.0	1749.1	6215.2	7964.3
		1.0	2293.0	6263.5	8557.5
	R	0.0	2600.3	7772.6	10372.9
		0.0	2943.5	8459.0	11402.5

[a] Values not corrected for % recovery.

Appendix 5 -- Comparison of percent coefficient of variation of B_6 vitamer content in foods determined by HPLC using external and internal standardizations

Food	Vendor	Calibration Method	PAM	PAL	POL	Total B_6
			Coefficient of variation (%)[a]			
Bread	K	ext	0.0	22.9	12.0	15.5
		int	0.0	22.8	12.0	15.0
	R	ext	0.0	28.0	24.0	25.7
		int	0.0	17.4	13.0	14.9
	Average	ext	0.0	23.8	39.0	32.1
		int	0.0	18.9	35.1	28.4
Milk	K	ext	15.6	30.0	10.9	2.9
		int	17.2*	31.9*	9.0	3.1*
	R	ext	0.0	5.9	30.5	14.4
		int	29.3*	35.6*	0.3	14.4
	Average	ext	16.3	24.5	18.6	10.6
		int	32.8*	41.2*	6.5	16.2
Peas	K	ext	120.0	2.5	16.1	10.9
		int	140.0*	19.0*	5.5	5.1
	R	ext	0.0	33.7	0.5	1.4
		int	0.0	8.7	6.0*	6.7*
	Average	ext	250.0	24.5	18.6	10.6
		int	166.7	21.1	15.6	16.6*

[a] Computed from $\frac{SD}{\overline{X}} \times 100$, SD and $\overline{X}$ values obtained from tables 10 & 12.

* (Percent coeff. of variation)$_{\text{int. standardization}}$ > (Percent coeff. of variation)$_{\text{ext. standardization}}$.

Appendix 6 -- Individual values for % recovery of B_6 vitamers in selected foods by HPLC

Food	Vendor	Spiked at beginning			Spiked before injection		
		PAM	PAL	POL	PAM	PAL	POL
Bread	K	97.0	-13.1	10.9	108.9	85.4	80.3
		83.7	4.8	11.8	113.7	72.4	62.2
	R	90.4	-10.7	67.4	126.5	65.4	92.9
		67.6	40.6	65.6	91.1	55.3	66.0
Milk	K	82.7	-3.6	7.3	130.1	110.2	109.0
		50.4	4.8	0.0	134.5	120.1	113.6
	R	81.8	0.0	16.4	76.8	56.8	97.3
		56.1	10.7	3.7	69.6	49.7	98.1
Peas	K	93.2	35.8	94.9	104.9	108.5	96.6
		59.9	70.5	96.0	111.8	106.0	92.1
	R	97.0	66.8	134.9	91.3	70.4	94.1
		58.9	72.1	62.0	67.2	96.8	96.6

Appendix 7 -- Individual values for GC-EC assay of B_6 vitamers in selected foods using external standardization[a]

Food	Vendor	PAM	PAL	POL	Total B_6
		(µg/100g wet wt)			
Bread	K	481.0	387.4	1210.8	2079.2
		332.2	290.6	871.6	1494.4
	R	241.0	104.1	599.2	944.3
		296.8	154.0	1152.0	1602.8
Milk	K	1005.8	549.3	5029.1	6584.2
		898.2	270.8	4126.4	5295.4
	R	1323.2	174.4	6506.7	8004.3
		2005.0	152.6	5728.4	7886.0
Peas	K	765.2	390.5	6260.5	7416.2
		1217.4	395.7	4391.3	6004.4
	R	1228.8	178.4	8097.9	9505.1
		1483.1	138.9	8662.9	10284.9

[a] Values not corrected for % recovery.

Appendix 8 -- Individual values for microbiological assay of B_6 vitamers in selected foods

Food	Vendor	PAM (µg/100g wet wt)	PAL (µg/100g wet wt)	POL (µg/100g wet wt)	Total B_6 (µg/100g wet wt)
Bread	K	37.2	75.5	30.0	142.8
		58.0	78.9	33.0	169.9
		59.0	91.4	26.9	177.3
		52.0	90.5	30.2	172.7
	R	44.0	56.9	17.7	118.6
		40.9	53.5	17.9	112.3
		36.9	61.7	16.1	114.7
		54.1	55.7	19.3	129.1
Milk	K	113.6	304.3	98.8	516.7
		113.6	296.4	77.1	487.1
		141.6	486.1	86.3	714.0
		136.3	480.9	79.1	696.3
	R	75.4	360.9	57.4	493.7
		69.2	339.2	60.2	468.6
		84.5	426.5	73.5	484.5
		90.5	419.9	75.5	585.9
Peas	K	14.0	2.4	18.1	34.2
		12.7	2.2	13.4	28.5
		14.4	2.5	14.5	31.4
		13.7	2.6	11.8	28.1
	R	16.3	3.5	17.5	37.3
		21.7	2.7	19.4	43.8
		18.4	4.1	17.3	39.8
		22.3	3.7	14.5	40.5

Appendix 9 -- Percent recovery of standards of B_6 vitamers from derivatization treatment for GC-EC analysis

Derivatization treatment	PAM	PAL	POL
	Recovery %		
I	71.4	67.1	69.8
	116.0	75.5	64.6
II	100.0	75.0	61.2
	70.0	67.0	64.7
Average[a]	89.4	71.2	65.1
	±22.5	±4.7	±3.5

[a] $\bar{X} \pm$ SD from two separate derivatization treatments.

Appendix 10 -- Comparison of percent coefficient of variation of B_6 vitamer content in foods as determined by HPLC, GC-EC and S. uvarum methods

Food	Source	Assay	PAM	PAL	POL	Total B_6
			Coefficient of variation (%)[a]			
Bread	K	HPLC	0.0	22.9	12.0	15.5
		GC-EC	25.9	20.2	23.0	23.1
		S. uvarum	19.6	9.6	10.0	9.4
	R	HPLC	0.0	28.0	24.0	25.7
		GC-EC	14.6	44.8	44.6	36.6
		S. uvarum	20.4	7.4	9.0	6.2
Milk	K	HPLC	15.6	30.0	10.9	2.9
		GC-EC	8.0	48.0	13.9	15.3
		S. uvarum	11.7	27.0	11.5	19.6
	R	HPLC	0.0	5.9	30.5	14.4
		GC-EC	29.0	9.4	9.0	1.0
		S. uvarum	11.9	18.5	11.9	10.4
Peas	K	HPLC	120.0	2.5	16.1	10.9
		GC-EC	32.2	0.9	24.8	14.9
		S. uvarum	52.5	8.3	24.6	9.1
	R	HPLC	0.0	33.7	0.5	1.4
		GC-EC	13.2	17.6	4.8	5.6
		S. uvarum	14.2	20.0	11.2	6.7

[a] Computed from $\frac{SD}{\bar{X}} \times 100$, SD and $\bar{X}$ values obtained from tables 8, 14, and 15.

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Comparison of high performance liquid chromatographic, gas liquid chromatographic, and Saccharomyces uvarum methods for the determination of B_6 compounds in selected foods.

by

Kim L. Lim

(ABSTRACT)

A high performance liquid chromatographic (HPLC) system consisting of an acetonitrile/phosphate buffer, a Spherisorb ODS column, and UV detector was used to separate the B_6 compounds pyridoxol, pyridoxal, pyridoxamine, and 4'-deoxypyridoxine. A gas chromatography (GC) equipped with a ^{63}Ni electron capture (EC) detector was used to separate the N-methyl-bis-trifluoroacetamide derivatives of the B_6 compounds on a 1.54m x 2mm i.d. glass column packed with 10% SP2100 on Supelcoport 90-100 mesh at 125°C and an inlet pressure of 40 psig. Clean and successful separations of all the B_6 forms were obtained by HPLC and GC-EC. Total B_6 values as determined by HPLC and GC-EC for all 3 foods were higher than the corresponding total B_6 values as determined by S. uvarum assay. Several of the B_6 vitamer values for foods obtained by GC-EC agree with corresponding values obtained by HPLC. The HPLC method seemed to be the most satisfactory of the 3 methods for the quantitation

of B_6 vitamers in foods and has the following advantages: increased sensitivity, method simplicity, and good precision.