MICROBIOLOGICAL ASSAY VARIABLES FOR DETERMINING VITAMIN B-6 CONTENT OF CHICKEN MUSCLE

by

PAULA FELDER MARMET

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Approved by:

Jane Bowers
Major Professor

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INTRODUCTION

Vitamin B-6 occurs naturally in food as pyridoxine (PN), pyridoxal (PL) and pyridoxamine (PM). The microbiological assay using the yeast Saccharomyces uvarum (ATCC 9080) is the usual method of choice for determination of vitamin B-6 content of food products (AOAC, 1984). However, several methodology variables are suspected to affect resulting vitamin B-6 values including length of time for incubation, type of medium and composition of standard curve. Various studies have demonstrated that the growth response of S. uvarum to PM was considerably less than the growth response to PN or PL (Parrish et al., 1955; MacArthur and Lehman, 1959; Gregory, 1982; and Guilarte et al., 1980). Therefore, an assay for total vitamin B-6 determination based on the growth response of S. uvarum may be subject to inaccuracy depending on the amount of PM present in the sample as compared with the amount of PM in the reference standard curve. The degree of inaccuracy is believed to be related to the amount of PM in the sample being analyzed (Guilarte et al., 1980).

The focus of this research was to study two lengths of time for incubation, two types of media, three standard curves of different compositions and three methods of constructing standard curves for calculating vitamin B-6 content of chicken muscle. Ion exchange chromatography to separate the individual forms of the vitamin was carried out in order to estimate the

proportion in which the three forms of the vitamin occur in chicken muscle.

LITERATURE REVIEW

Media. The basal medium described in the AOAC (1984) method is composed of a fermentable sugar (dextrose), amino acids, inorganic salts, vitamins and a buffer. Parrish et al. (1956 and 1955) studied the effects of amino acids in the basal medium on the growth response of S. uvarum in the presence of vitamin B-6. When casein hydrolysate prepared in the laboratory was used in the medium, more variation in the yeast response was observed than when a synthetic mixture of amino acids was used. Further investigation revealed that five of the amino acids contained in a synthetic amino acid mixture used to simulate casein hydrolysate caused an increase in growth response when present and a decrease in growth response when absent. These included isoleucine, histidine, methionine, tryptophane and valine. The standard medium (AOAC, 1984) includes both the five amino acids which Parrish et al. (1956) determined to be essential and a laboratory prepared casein hydrolysate solution.

Several researchers have more recently substituted Pyridoxine-Y-Medium, a synthetic dehydrated medium (Difco Laboratories) for convenience as it takes only a few minutes to prepare (Gregory, 1982 and Guilarte et al., 1980). Components of this medium are patterned after the formulation of the AOAC medium, but amino acid composition is modified to include only bacto-asparagine, L-histidine hydrochloride, DL-methionine, DL-tryptophane, DL-isoleucine and DL-valine (Difco, 1977).

Growth response of the test organism. The yeast Saccharomyces uvarum (ATCC 9080) traditionally has been the most widely accepted organism for use in the microbiological assay for measuring the free biologically active forms of vitamin B-6 despite the differential growth response to the three forms. Polansky (1980) reported less growth response to PM than to PL or PN (Fig. 1). Parrish et al. (1955) reported the growth response of this yeast to PM was 50 to 60% of the response to PN or PL. More recently, Guilarte et al. (1980) reported similar findings. At concentrations of 2-10 ng/vial, PN produced the greatest growth stimulation; growth response to PL and PM was less than 50% of that to PN. At the higher concentrations (10 ng/vial) the growth response of S. uvarum to PL and PM was 80% of that of PN, suggesting the differential growth response to the three forms of vitamin B-6 could be dose dependent. Gregory (1982) also reported lower growth response to PM when a range of concentrations more typical in food analysis (0-10 ng vitamin/tube) was used. His results demonstrated a strong dependence of the response of the organism to the concentration of PM. Lower concentrations produced greater differences in the growth response than was apparent at higher concentrations.

Length of incubation. Parrish et al. (1955) compared growth response of S. uvarum to the three vitamers in the microbiological assay when incubated for varying lengths of time. After 22 hr of incubation, the response to PN and PL

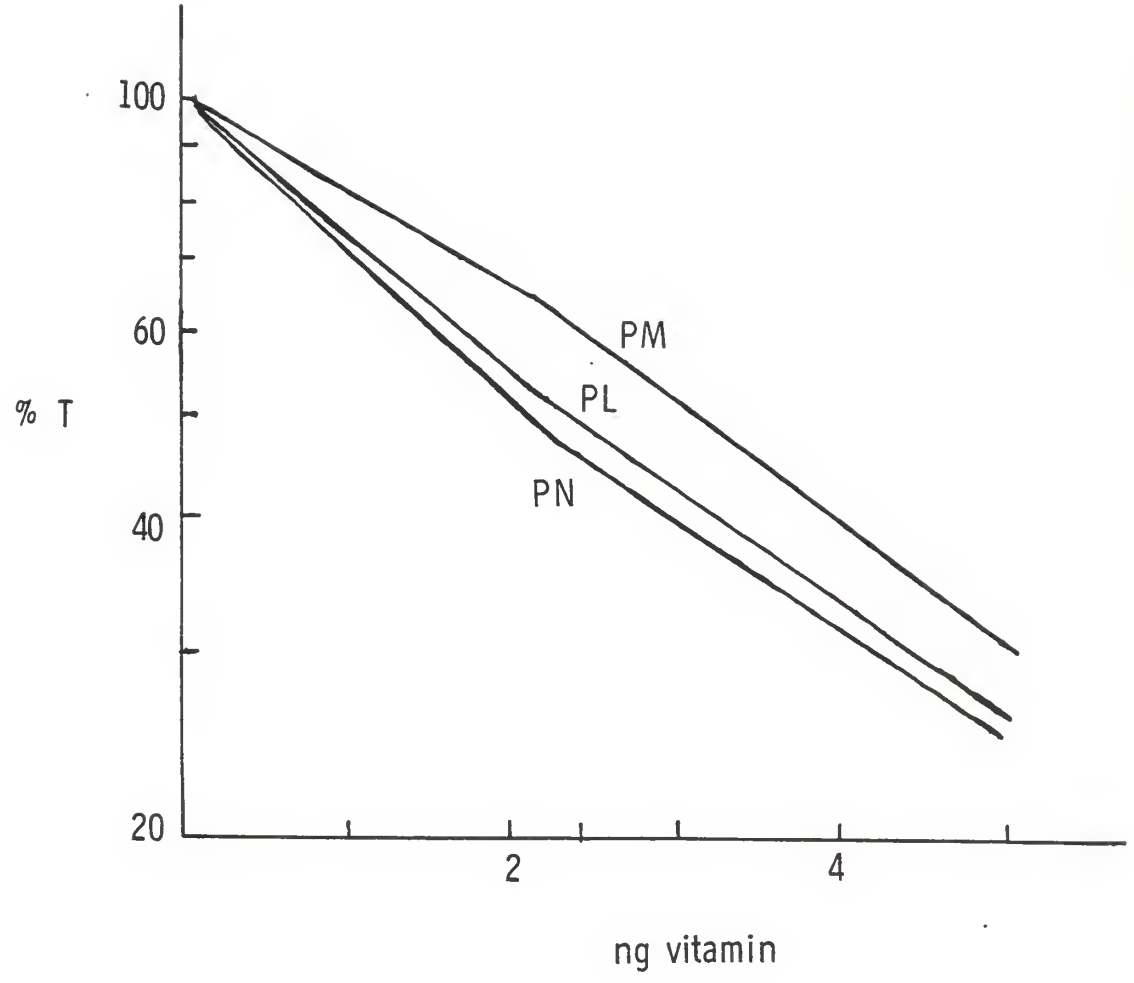

Figure 1. Typical growth response of S. uvarum to the three forms of vitamin B-6. (Polansky, 1980)

was considered equal and maximum; this response held constant through a 28 hr incubation period. Additionally, he reported that growth response of the organism to PM during this time period was considerably less; maximum growth response to PM was not approached until 36 hr. However, yeast response to PN and PL was erratic and stimulated when allowed to incubate beyond 28 hr. These researchers concluded that the incubation time should not be less than 22 hr, nor more than 28 hr when standard solutions with concentrations of 1 ng/ml is used.

Composition of reference standard curve. The accepted method (AOAC) for vitamin B-6 determination in food samples is based on the microbiological assay of each form of vitamin B-6 after separation on an ion-exchange column. In this case the amount of vitamin in each sample fraction is interpolated from a standard curve of each form of the vitamin. When assaying for total vitamin B-6 in a sample, the composition of the reference standard curve seems to vary from one study to another. Daoud et al. (1977and 1973) used a standard curve containing PN to determine vitamin B-6 content of garbanzo beans. The composition of the standard curve in this case seems reasonable since plants contain primarily the PN form. Bowers and Craig (1978) used a mixture of the three forms to determine the amount of total vitamin B-6 in turkey muscle. In this case the composition of the standard curve was based on values determined from column separation indicating the proportion of the vitamin B-6 components of turkey muscle to

be approximately 80% PM, 10% PN and 10% PL.

Hamaker (1983 and 1985) also used a PN standard curve to determine total B-6 in milk samples. Polansky (1980) reported the value for a non-chromatographed total B-6 value of milk which contains comparatively high proportion of PM to be approximately 37% lower than that determined by column separation and assayed for the individual forms of the vitamin.

Separation of B-6 vitamers. Because of the differential growth response of the test organism to the three forms of vitamin B-6, procedures were developed to separate PN, PL and PM and determine their amounts individually to obtain more accurate vitamin B-6 values. After a lengthy investigation of cation exchange resins, Dowex AG 50W-X8 (100-200 mesh) in the sodium form, was found to give a complete and individual separation of the three forms (MacArthur and Lehmann, 1959). This procedure involved placing a desired amount of standard solution on the column; the column was then washed and PL, PN and PM were eluted in succession. Amounts of vitamer in each fraction were measured with a spectrophotofluorometer.

Subsequent studies adapted this procedure to the vitamin B-6 assay of food and biological products (Toepfer and Lehmann, 1961 and Toepfer and Polansky, 1970): Ground food samples were extracted by autoclaving in the presence of 0.055N HCl (5 hr for animal products) at 15 lb steam pressure. The extracts were adjusted to pH 4.5, made to volume, filtered, and an aliquot placed on the column. After washing the column (Dowex

AG 50W-X8, potassium form) to remove salts and other materials from the extract, the PN, PL and PM were removed in succession. The chromatographed fractions were subjected to the microbiological assay procedure and amount of each form of the vitamin was determined from interpolation from a standard curve. Both teams of researchers reported mean recoveries of 97% or greater when individual forms of the vitamin were placed on the column and mean recoveries of 98% or greater when a mixture of all three forms was placed on the column. Their procedure, with few improvements, still is used today (AOAC, 1984).

A collaborative study (Toepfer and Polansky, 1970) involving nine laboratories was undertaken to determine content of individual forms of the vitamin in lima beans, whole wheat flour, dried beef liver and enriched bread. The vitamers were separated by ion-exchange chromatography and content of vitamin in samples determined by interpolation from a standard curve of each vitamer after microbiological assay. The mean values for total vitamin B-6 (interpolated from a PN standard curve) and the three components were significantly different for beef liver only. This was explained by the differential growth response of the test organism to PM; the amount of PM in dried beef is proportionately higher than in the other samples analyzed.

EXPERIMENTAL PROCEDURES

Two studies were conducted: 1) The first study examined four variables which are suspected to influence values obtained in the microbiological assay of total B-6 in chicken muscle. 2) The second study investigated the effect of altering the pH of the buffer used to elute the PL fraction of the sample during ion-exchange chromatography.

Preparation of samples. Cooked chicken samples were prepared as described by Toepfer and Polansky (1970) and AOAC (1984). Four chicken breasts were purchased from a fast-food fried chicken outlet on four separate occassions. Skin, bone and coating material were removed and muscles were homogenized for one minute with a Sunbeam food processor. Duplicate 2-gm samples of breast muscle were digested with 200 ml 0.055N HCl for 5 hr in an autoclave at 121°C. The extract was cooled, pH adjusted to 4.5, diluted to 250 ml and filtered. Extracts were frozen in opaque bottles at -4°C and held no longer than two months. For each assay, sample extracts were thawed and diluted to contain approximately 1 ng of vitamin B-6/ml and assayed at the 0.5, 1.0, 2.0, 4.0 and 5.0 ml levels.

Composition of standard curves. Three standard curves of different composition were prepared in duplicate with a concentration range of 0.5 - 5.0 ng/tube. Standard solution variations were: 1) equal portions of PL, PN and PM; 2) eight parts PM, one part PN and one part PL; and 3) PN only.

Test organism. Saccharomyces carlsbergensis (ATCC 9080)

was maintained by weekly transfers on wort agar slants. To prepare inoculum for assay, cells were incubated on agar for 24 hr at 30°C. These cells were transferred under aseptic condition to liquid broth culture tubes which contained 5 ml of vitamin B-6-free basal medium and 5 mls of mixed PN, PL, and PM solutions (1 ng/ml). Liquid broth culture was sterilized in capped test tubes for 10 min at 121°C in an autoclave before inoculation. Inoculum was incubated overnight at 30°C. Cells were separated by centrifugation for 9 min at 2500 RPM and the liquid decanted. Cells were resuspended in 10 ml 9% saline, centrifuged 9 min at 2500 RPM and liquid decanted. Cells suspended in the third 10 ml saline rinse were used as assay inoculum.

Assay inoculum. Cells from the liquid broth culture which had been rinsed 3 times were suspended in 10 mls of sterile 9% saline and adjusted to measure 95% transmission at 550 nm in a Bausch and Lomb spectrophotometer. Using a sterile pipette, one drop of this solution was placed in each tube to be assayed. Tubes were incubated at 30°C on a rollo-drum rotator.

Length of incubation. Each assay was prepared in duplicate to allow comparison of optimum vs. extended incubation times. When the prepared Difco medium was used, the optimum incubation time was 26 hr; extended time was 28 hr. When the standard medium (Toepfer and Polansky, 1970) was used, the optimum incubation time was 22 hr and extended time was 24 hr.

Media preparation. Two types of media were compared. A dehydrated prepared medium from Difco laboratories, Pyridoxine-Y-Medium, which was rehydrated on the same day as the assay was prepared, and the standard medium described by Toepfer and Polansky (1970). Dehydrated Bacto Vitamin-free casamino acids (Difco laboratories) were used in place of the laboratory prepared casein hydrolysate in the standard medium. The primary difference between the medias was in the amino acid content. The Difco medium contained bacto-asparagine, L-histidine hydrochloride, DL-methionine, DL-tryptophane, DL-isoleucine and DL-valine. The standard medium contained dehydrated vitamin-free acid hydrolyzed casein in addition to those amino acids contained in the Difco medium.

Calculations. Turbidity (percentage transmission) was measured at 550 nm with a Bausch & Lomb spectrophotometer. Concentration of total vitamin B-6 in the samples were interpolated using three different methods for plotting standard curves:

1) In the first method, "hand", the standard procedure was followed (Toepfer & Polansky, 1970). Data points were plotted on semi-log paper as percentage transmission (%T) against ng of standard. A line to best fit the data points was drawn with a straight edge and concentration of sample was interpolated from this line.

2) In the second method, "linear", a computer calculated linear equation ($y = mx + b$) was used to construct a least

squares line from the data points. Concentration of vitamin B-6 in the sample was computer calculated by solving for X (concentration of vitamin) based on Y (Log %T).

3) The third method of calculation, "quadratic", used a computer calculated quadratic equation ($y = a + bx + cx^2$) to construct the standard curve. Concentrations of vitamin B-6 in sample was computer calculated by solving the quadratic equation:

$$x = -\hat{B}_1 \pm \frac{\sqrt{\hat{B}_1^2 \quad 4(\hat{B}_2)\ (\hat{B}_o - Y)}}{2(\hat{B}_2)}$$

for X (vitamin content) given Y (Log %T). Other variations of computer calculated values are presented in the Appendix.

Analysis of data. Values for vitamin content of cooked chicken muscle as determined by microbiological assay were subjected to analyses of variance. Sources of variation were identified and the following design used:

Source of variation	df
Replication	3
Medium	1
Length of incubation (Hr)	1
Medium vs. Hr	1
Replication vs. Medium vs. Hr	9
Standard	2
Medium vs. Standard	2
Hr vs. Standard	2
Medium vs. Hr vs. Standard	2
Error	24
Corrected Total	47

Statistical design for analysis of data from other variations of computer calculated values are located in the Appendix.

Column separation of vitamers. Dowex AG 50W-X8 (100-200 mesh) was activated and vitamer separation was carried out as described by Toepfer and Polansky (1970) and AOAC (1984). In an attempt to improve column separation of PL from PN, we adjusted the pH of the solution used to elute PL from the column. Eluants of pH 6.0 and 6.2 were compared for: 1) standard solution containing 10 ug PL only; 2) standard solution containing 10 ug PL, 10 ug PN and 10 ug PM; 3) 100 ml of the filtered sample extract. A duplicate set of columns was prepared and the separation procedure carried out substituting 0.04 M potassium acetate buffer with pH adjusted to 6.2 rather than 6.0 to elute the PL from the column.

Turbidity (percentage transmission) was measured at 550 nm with a Bausch & Lomb spectrophotometer. Concentration of each form of the vitamin was interpolated from each of the three standard curves using the "hand" method of calculation.

RESULTS AND DISCUSSION

Methodology variables for vitamin B-6 microbiological assay tested included two lengths of time for incubation, two types of media, three standard curves of different compositions and three methods of constructing standard curves for calculating B-6 values in chicken muscle.

Length of incubation time. Extending the incubation time of the assay by two hours did not significantly affect determined B-6 values regardless of which type media was used (Table 1). Parrish et al. (1955) demonstrated that yeast response to PN and PL was erratic and stimulated when allowed to incubate beyond the optimum incubation time. Toepfer and Polansky (1970) indicated that 22 hr was the optimum incubation time when using the standard medium and assaying standard solutions at levels of 1 ng vitamin/ml. Since no appreciable additional growth was observed when the expected optimum incubation time was extended, we conclude that optimum growth was achieved but not exceeded in the case of either medium.

Effect of media. When the standard medium (AOAC, 1984) was used, determined values for total vitamin B-6 content tended to be higher in all cases than when the Difco medium was used in the microbiological assay (Table 2). The observed difference was significant at the 10% level, but this was true only when the determined values were calculated by the "hand" method. Trends were the same for "linear" and "quadratic" methods, but the difference in observed levels was not

significant when determined vitamin B-6 values were calculated by the "linear or "quadratic" methods. The tendency for increased growth response observed when using the standard medium is most likely explained by the difference in amino acid content. (See Appendix for media composition) Both medias contained the five amino acids which Parrish et al. (1956) determined to be essential for optimum yeast growth, but the vitamin-free casamino acids in the standard medium includes four additional amino acids not present in the Difco medium. These additional amino acids are glutamic, phenylalanine, threonine and tyrosine. They were observed by Parrish et al. (1956) to stimulate yeast growth when present, but did not produce a negative growth effect when eliminated from the synthetic mixture.

Table 1. Vitamin B-6 (mg/100g) content of chicken muscle using two lengths of incubation time[1].

	Approximate optimum time	Approximate optimum + 2 hr
Method of calculation		
Hand	0.57235_a	0.57581_a
Linear	0.53964_a	0.53103_a
Quadratic	0.59487_a	0.54681_a

[1]Means of 4 replications
Means with same subscript are not significantly different. $P<0.05$

Table 2. Vitamin B-6 (mg/100g) content of chicken muscle using two types of media[1].

	Standard (AOAC, 1984)	Difco
Method of calculation		
Hand	0.60581_a	0.54235_b
Linear	0.55220_a	0.51848_a
Quadratic	0.59967_a	0.54202_a

[1]Means of 4 replications.
Means with same subscript are not significantly different.
$P<0.10$

Composition of standard curve. A significant difference among determined B-6 values was observed for values calculated from standard curves of different compositions (Table 3). Vitamin B-6 values calculated from the standard curve composed of PM, PL and PN in proportions of 8:1:1 were significantly higher than those from a standard curve of equal proportions of PM, PN and PL or from a standard curve of PN only. Vitamin B-6 values calculated from standard curves of equal proportions tended to be greater than those calculated from standard curves composed of PN only, but the difference was not significant.

Table 3. Vitamin B-6 (mg/100g) content of chicken muscle calculated from three standard curves using three methods.[1]

	PN	PM:PN:PL 1 :1 :1	PM:PN:PL 8 :1 :1
Method of calculation			
Hand	0.48112_b	0.52416_a	0.71697_a
Linear	0.42242_a	0.49562_a	0.68797_a
Quadratic	0.45446_a	0.52046_a	0.73762_a

[1]Means of 4 replications.
Means with same subscript are not significantly different.
$P<0.05$

Studies conducted by Parrish et al. (1955) demonstrated that S. uvarum requires more time to utilize PM than it does the other two forms of the vitamin. Figure 2 illustrates the observed differential growth response of the test organism to the three standard curves tested. This fact probably best explains why interpolating vitamin content of the sample from the standard curve containing the greater concentration of PM resulted in the higher determined value. Toepfer and Lehmann (1961) and Rabinowitz and Snell (1948) have determined PM to be the predominant form of vitamin B-6 in animal tissue. Therefore, it would be expected that the growth response of the test organism in a standard curve composed of a greater

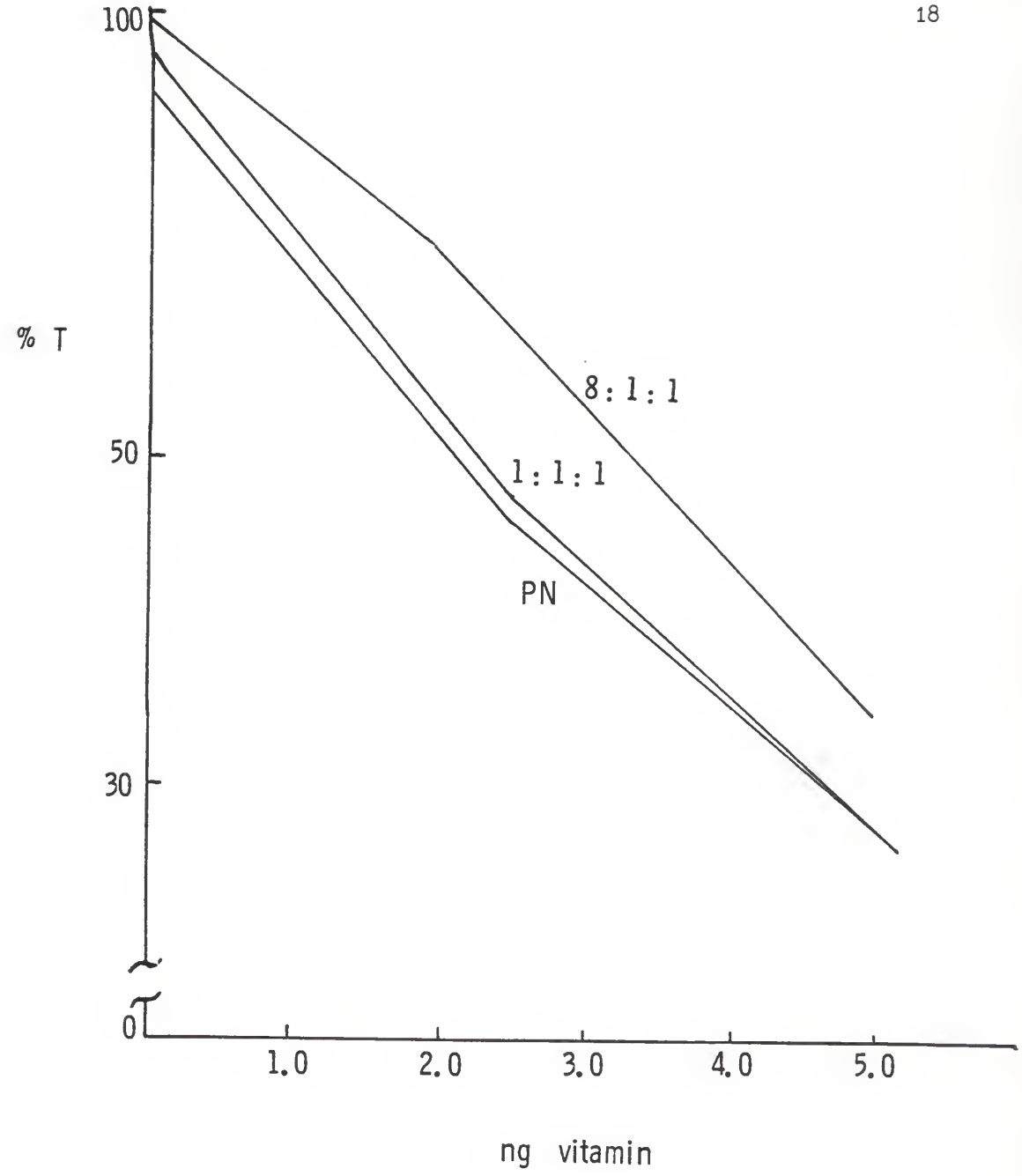

Figure 2. Typical growth response of S. uvarum to three standard curves of different composition.

proportion of PM would better simulate the growth response in the extract of an animal tissue such as chicken muscle.

Method of calculation. In some cases, the method of calculation used to construct the standard curves and interpolate vitamin content of the sample affected determined B-6 values. Values determined from standard curves which were computer calculated from linear or quadratic equations were generally lower than those which were determined from a standard curve drawn by hand. In the case of the PN standard curve, the determined vitamin B-6 values interpolated from the standard curve using the "hand" method of calculation were significantly different than values derived using either the "linear" or "quadratic" methods (Table 3).

Due to the curvilinear pattern of the growth curve of the organism, both methods involving computer calculations to construct standard curves involved taking the log of the %T to simulate the growth pattern that results when plotting %T by the "hand" method on semi-log paper. No significant difference was observed between determined vitamin B-6 values obtained from the "hand" method and the computer calculated methods of calculation. However, it would seem that the computer calculated methods would eliminate the potential variation which may occur between one individual and another in constructing standard curves and interpolating vitamin B-6 content when using the "hand" method.

Column separation. Efforts to separate the three forms of

vitamin B-6 in a standard solution as described by Toepfer and Polansky (1970) did not achieve as complete a recovery as that reported by Polansky (1980). Observed recovery of PL when only this form of the vitamin was placed on the column was 89.9% compared with 97±4.6% reported by Polansky (Table 4). Personal communication with Ms. Polansky revealed that she had experienced varying degrees of recovery when resin from different manufacturing lot numbers was tested. Resin from some lot numbers seemed to produce more complete recovery than others. At her suggestion, pH of the 0.04 M KOAC buffer used to elute the PL from the column was adjusted. Increasing the pH of this buffer to 6.2 resulted in a higher recovery of PL when it was placed on the column individually. However, the difference in PL recovery was not significant nor was the difference in percentage total recovery significant.

Adjusting the pH of the 0.04 M KOAC buffer did not significantly affect the determined values obtained for individual forms of the vitamin contained in the chicken muscle sample. The trend was toward a higher determined value when the pH was increased from 6.0 to 6.2, but the difference was not significant.

Observed results of column separation of the individual forms of the vitamin and microbiological assay indicate that the tissue content of vitamin B-6 in chicken muscle is approximately 70% PM, 20% PN and 10% PN. This is similar to the approximate proportions of the three vitamers in turkey

muscle observed by Bowers and Craig (1978).

Table 4. Recovery of individual B-6 vitamers with ion exchange chromatography.[1]

Fractions eluted	0.04M KOAC pH=6.0	0.04M KOAC pH=6.2
Standard solution of PL		
0.01M KOAC	---	---
0.04M KOAC	6747	6219
0.10M KOAC	2031	2686
KCL-K_2HPO_4	128	156
Total ng eluted	8906	9061
Total ng placed on column	10,000	10,000
Recovery (%)	89	90.6
Standard solution of PL:PN:PM		
0.01M KOAC	15	43
0.04M KOAC	9852	7544
0.10M KOAC	8284	10,775
KCL-K_2HPO_4	10,893	10,411
Total ng eluted	29,044	28,773
Total ng placed on column	30,000	30,000
Recovery (%)	96.8	95.9
Chicken sample extract		
0.01M KOAC	.0008	---
0.04M KOAC	.0868	.0891
0.10M KOAC	.0496	.0546
KCL-K_2HPO_4	.2600	.2985
Total vitamin (mg/100g) eluted	.3972	.4422

[1]Means of 4 replications.

SUMMARY

Methodology variables in the microbiological assay for determination of vitamin B-6 content of chicken muscle were tested. Those included length of time for incubation, type of media, composition of standard curve and method of constructing standard curves for calculating vitamin B-6 content. Values interpolated from standard curves containing 80% pyridoxamine, 10% pyridoxal and 10% pyridoxine were significantly higher ($P<.05$) than those interpolated from standard curves containing equal amounts of the three forms of vitamin B-6 or from those containing pyridoxine only.

Extending the estimated optimum incubation time by two hours did not significantly affect determined B-6 values. Estimated optimum incubation time when using Difco media was determined to be 26 hr; estimated optimum incubation time when using the standard AOAC medium was determined to be 22 hr when standard solution concentrations were 1 ng/ml. Determined vitamin B-6 values of assays using the standard AOAC medium were higher than those determined from assays using the prepared Difco medium. Difference was significant at the 10% level for the "hand" method of calculation.

Vitamin B-6 values determined from standard curves which were computer calculated from linear or quadratic equations were generally lower than those which were determined from a standard curve drawn by "hand"; however, the difference was not significant except when the standard curve contained PN

only.

Observed results of column separation of the individual forms of the vitamin indicate that the tissue content of vitamin B-6 in cooked chicken muscle is approximately 70% PM, 20% PN and 10% PL.

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APPENDIX

GLOSSARY OF TERMS AND ABBREVIATIONS

BR Four chicken breast muscles.

DUP Two determinations for each breast muscle.

Hand One of seven methods of calculation explained in the text and referred to as "hand".

HR Length of time of incubation:
Difco Media; HR 1 = 26 hr
HR 2 = 28 hr

Standard Media; HR 1 = 22 hr
HR 2 = 24 hr

Linear 1 A varaiation of the "linear" method of calculation explained in the text. The computer calculated least squares line was constructed from the data points obtained from tubes containing standard solution in the concentration range of 1.0 - 5.0 ng/ml. Concentration of vitamin B-6 in the sample was computer calculated by solving for X (concentration of vitamin) based on Y (%T).

Linear 2 A variation of the "linear" method explained in the text. The computer calculated least squares line was constructed from data points which excluded tubes of standard solution which measured > 85% T. Concentration of vitamin B-6 in the sample was computer calculated by solving for X (concentration of vitamin) based on Y (%T).

Linear 3 The method of calculation explained in the text and referred to as "linear".

MED Two types of media: Difco media = 1
Standard media = 2

Quadratic 1 A variation of the "quadratic" method of calculation explained in the text. Data points obtained from tubes containing standard solutions with concentration range of 1.0 - 5.0 ng/tube were used in the computer calculated quadratic equation to construct the standard curves. Concentration of vitamin B-6 in the sample was computer calculated by solving the quadratic equation for X (vitamin content) given Y (%T).

Quadratic 2 A variation of the "quadratic" method of calculation explained in the text. Data points with > 85% T were excluded when constructing the standard curve using the quadratic equation. Concentration of vitamin B-6 in sample was computer calculated by solving the quadratic equation for X (vitamin content) given Y (%T).

Quadratic 3 The method of calculation explained in the text and referred to as "quadratic".

STD Three standard curves of different composition used to interpolate vitamin B-6 values:

1 = PN only
2 = 80% PM, 10% PL, 10% PN
3 = PN, PM, and PL in equal portions

Analysis of data using 7 methods of calculation.

Values for vitamin content of cooked chicken muscle as determined by microbiological assay using seven methods of calculation were subjected to analysis of variance. Variations of computer calculated values were identified and the following design used:

Source of variation	df
Replication	3
Media	1
Length of incubation (Hr)	1
Media vs. Hr	1
Replication vs. media vs. hr	9
Standard	2
Media vs. Standard	2
Hr vs. Standard	2
Media vs. Hr vs. Standard	2
Replication vs. Media vs. Hr vs. Standard	24
Method of calculation (Method)	6
Media vs. Method	6
Hr vs. Method	6
Media vs. Hr vs. Method	6
Method vs. Standard	12
Media vs. Method vs. Standard	12
Hr vs. Method vs. Standard	12
Media vs. Hr vs. Method vs. Standard	12
Error	216
Corrected total	335

Means of 4 replications determined by 7 methods of calculation are presented in Tables 5, 6 and 7.

Table 5. Vitamin B-6 (mg/100g)[1] content of chicken muscle using two lengths of incubation time.[1]

	Approximate optimum time	Approximate optimum time plus 2 hr
Method of calculation		
Hand	0.57235_a	0.57581_a
Linear 1	0.53098_a	0.53504_a
Linear 2	0.56760_a	0.52842_a
Linear 3	0.53964_a	0.53103_a
Quadratic 1	0.52163_a	0.52619_a
Quadratic 2	0.54660_a	0.53683_a
Quadratic 3	0.59487_a	0.54681_a

[1]Means of 4 replications
$P<0.05$

Table 6. Vitamin B-6 (mg/100g) content of chicken muscle using two types of media.[1]

	Standard (AOAC, 1984)	DIFCO
Method of calculation		
Hand	0.60581_a	0.54234_b
Linear 1	0.56199_a	0.50402_a
Linear 2	0.58981_a	0.50622_a
Linear 3	0.55220_a	0.51848_a
Quadratic 1	0.56197_a	0.48584_b
Quadratic 2	0.57650_a	0.50694_a
Quadratic 3	0.59967_a	0.54202_a

[1]Means of 4 replications
P<0.10

Table 7. Vitamin B-6 (mg/100g) content of chicken muscle from three standard curves using three methods of calculation.[1]

	PN	PM:PN:PL 1 :1 :1	PM:PN:PL 1 :1 :1
Method of calculation			
Hand	$0.48112_{a,d,h}$	0.52416_{a}	0.71697_{a}
Linear 1	0.47139_{a}	0.49103_{a}	0.63661_{b}
Linear 2	$0.46473_{a,f}$	0.48570_{a}	$0.69360_{a,e}$
Linear 3	$0.42242_{e,f,g}$	0.49562_{a}	$0.68797_{a,e}$
Quadratic 1	$0.41316_{b,g}$	0.48827_{a}	$0.67029_{b,c}$
Quadratic 2	$0.41693_{b,c}$	0.50118_{a}	$0.70704_{a,c,d}$
Quadratic 3	$0.45446_{c,d,e,g,h}$	0.52046_{a}	$0.73762_{a,d}$

[1]Means of 4 replications
$P<0.05$

Table 8. Vitamin B-6 content (mg/100g) as determined using 7 methods of calculation.

BR	DUP	HR	STD	MED	HAND	LIN1	QUAD1	LIN2	QUAD2	LIN3	QUAD3
1	1	1	1	1	0.410	0.3472	0.4032	0.4097	0.4116	0.4133	0.4138
1	2	1	1	1	0.425	0.4454	0.3341	0.3414	0.3429	0.3472	0.3790
1	1	1	2	1	0.530	0.4336	0.5358	0.5419	0.5299	0.5290	0.5291
1	2	1	2	1	0.456	0.3824	0.4560	0.4671	0.4574	0.4576	0.4575
1	1	1	3	1	0.460	0.4560	0.4062	0.4560	0.4660	0.4670	0.4703
1	2	1	3	1	0.400	0.3849	0.3557	0.3849	0.3963	0.3988	0.4013
1	1	2	1	1	0.491	0.5168	0.3935	0.4008	0.4002	0.4096	0.4134
1	2	2	1	1	0.425	0.4570	0.3359	0.3471	0.3473	0.3535	0.3552
1	1	2	2	1	0.519	0.4380	0.5118	0.5008	0.5073	0.5125	0.5138
1	2	2	2	1	0.480	0.3974	0.4750	0.4551	0.4656	0.4691	0.4708
1	1	2	3	1	0.506	0.4266	0.3777	0.4266	0.4395	0.4447	0.4478
1	2	2	3	1	0.463	0.3742	0.3358	0.3742	0.3913	0.3943	0.3978
1	1	1	1	2	0.330	0.2974	0.3280	0.3446	0.3350	0.3372	0.3349
1	2	1	1	2	0.350	0.3168	0.3470	0.3617	0.3531	0.3572	0.3553
1	1	2	1	2	0.295	0.3293	0.2869	0.3736	0.3556	0.3619	0.3552
1	2	2	1	2	0.375	0.3489	0.3020	0.3908	0.3751	0.3831	0.3777
1	1	1	3	2	0.405	0.4323	0.3947	0.4323	0.4264	0.4293	0.4282
1	2	1	3	2	0.456	0.4492	0.4096	0.4493	0.4463	0.4500	0.4496
1	1	2	1	2	0.396	0.3649	0.3134	0.4048	0.3359	0.3266	0.2943
1	2	2	1	2	0.437	0.4169	0.3450	0.4505	0.3658	0.3588	0.3161
1	1	2	2	2	0.581	0.4048	0.3587	0.4390	0.3049	0.3652	0.3511
1	2	2	2	2	0.461	0.4558	0.3912	0.4839	0.3272	0.3984	0.3746
1	1	2	3	2	0.471	0.4730	0.4374	0.4030	0.3987	0.4019	0.4204
1	2	2	3	2	0.413	0.5192	0.4699	0.4377	0.4216	0.4255	0.4440
2	1	1	1	1	0.450	0.4596	0.3402	0.4596	0.3402	0.3377	0.3483
2	2	1	1	1	0.470	0.4938	0.3736	0.4938	0.3736	0.3708	0.3816
2	1	1	2	1	0.514	0.4585	0.5040	0.5158	0.5039	0.5070	0.5087
2	2	1	2	1	0.547	0.4756	0.5232	0.5387	0.5345	0.5335	0.5345
2	1	1	3	1	0.502	0.4077	0.3535	0.4077	0.4254	0.4262	0.4315
2	2	1	3	1	0.531	0.4373	0.3798	0.4373	0.4604	0.4585	0.4658
2	1	2	1	1	0.420	0.4417	0.3149	0.4417	0.3149	0.3233	0.3294
2	2	2	1	1	0.469	0.4808	0.3369	0.4808	0.3369	0.3463	0.2779
2	1	2	2	1	0.493	0.4131	0.4856	0.4746	0.4830	0.4859	0.4872
2	2	2	2	1	0.506	0.5311	0.5629	0.4680	0.5589	0.5592	0.5744
2	1	2	3	1	0.505	0.4352	0.3923	0.4352	0.4416	0.4453	0.4484
2	2	2	3	1	0.547	0.3725	0.4790	0.3725	0.4790	0.4552	0.4954
2	1	1	1	2	0.604	0.4806	0.5936	0.5490	0.5542	0.5098	0.6206
2	2	1	1	2	0.530	0.5617	0.4877	0.4641	0.4676	0.4331	0.5398
2	1	1	2	2	0.863	1.1481	1.1436	2.2917	1.4011	1.2150	1.4119
2	2	1	2	2	1.290	1.0720	1.0697	1.3069	1.3315	1.1240	1.3328
2	1	1	3	2	0.823	0.5452	0.4932	0.4582	0.4598	0.4602	0.4886
2	2	1	3	2	0.513	0.4744	0.4290	0.3811	0.4043	0.4045	0.4346
2	1	2	1	2	0.533	0.5459	0.4689	0.4312	0.4859	0.4852	0.4933
2	2	2	1	2	0.531	0.4704	0.3816	0.2967	0.4028	0.4034	0.4135
2	1	2	2	2	1.044	0.8578	0.8647	0.8499	0.8647	0.8877	0.8687
2	2	2	2	2	0.828	0.7691	0.7760	0.7585	0.7761	0.7987	0.7771
2	1	2	3	2	0.439	0.4637	0.5426	0.5234	0.5431	0.5475	0.5517
2	2	2	3	2	0.419	0.5174	0.4619	0.4321	0.4823	0.4693	0.4750
3	1	1	1	1	0.590	0.5772	0.5673	0.5770	0.5673	0.7282	0.5870
3	2	1	1	1	0.440	0.4035	0.2651	0.4035	0.3901	0.4429	0.4936
3	1	1	2	1	1.215	1.0074	0.9938	1.0022	0.9771	1.1360	1.0172
3	2	1	2	1	0.708	0.7005	0.6948	0.6755	0.6671	0.7120	0.6791
3	1	1	3	1	0.850	0.7542	0.8301	0.7409	0.8276	0.8606	0.9410
3	2	1	3	1	0.519	0.4344	0.4779	0.5065	0.5103	0.5332	0.5430
3	1	2	1	1	0.520	0.5405	0.5003	0.5733	0.5759	0.6702	0.7311
3	2	2	1	1	0.442	0.4192	0.2977	0.4339	0.3934	0.4467	0.4597

Table 8 continued.

JR	DUP	HR	STD	MED	HAND	LIN1	QUAD1	LIN2	QUAD2	LIN3	QUAD3
3	1	2	2	1	1.340	1.0205	1.0000	1.0135	1.2798	1.1524	1.1149
3	2	2	2	1	0.890	0.7400	0.7304	0.7093	0.7539	0.7609	0.7571
3	1	2	3	1	0.785	0.7586	0.8596	0.7586	0.8565	0.8566	0.8836
3	2	2	3	1	0.520	0.4834	0.5531	0.5471	0.5502	0.5679	0.5729
3	1	1	1	2	0.709	0.6680	0.5775	0.5069	0.5954	0.5934	0.6362
3	2	1	1	2	0.704	0.6067	0.6311	0.6598	0.5664	0.6026	0.7470
3	1	1	2	2	0.969	0.8585	0.9429	0.8585	0.9429	0.8917	1.0177
3	2	1	2	2	1.225	0.9575	1.1010	1.0107	1.2241	1.0967	1.0216
3	1	1	3	2	0.750	0.5637	0.6515	0.6193	0.6595	0.6481	0.6773
3	2	1	3	2	0.775	0.5818	0.7312	0.6003	0.7314	0.7145	0.8120
3	1	2	1	2	0.669	0.5937	0.6752	0.6557	0.5729	0.6003	0.6683
3	2	2	1	2	0.771	0.6930	0.7457	0.7462	0.7140	0.7194	0.7889
3	1	2	2	2	0.964	0.8473	0.9566	0.7999	1.0185	0.9257	0.9486
3	2	2	2	2	1.002	0.9234	1.0658	0.9365	1.1633	1.0401	1.0626
3	1	2	3	2	0.706	0.6312	0.7070	0.6748	0.6630	0.6417	0.6941
3	2	2	3	2	0.740	0.7257	0.7963	0.6363	0.7774	0.7536	0.8055
4	1	1	1	1	0.368	0.4633	0.3491	0.4633	0.3491	0.3433	0.3586
4	2	1	1	1	0.391	0.4943	0.3721	0.4943	0.3721	0.3674	0.6126
4	1	1	2	1	0.614	0.6173	0.6349	0.6159	0.6199	0.6210	0.6200
4	2	1	2	1	0.625	0.6272	0.6478	0.6329	0.6414	0.6434	1.4887
4	1	1	3	1	0.426	0.4081	0.3878	0.4081	0.4216	0.4241	0.4286
4	2	1	3	1	0.417	0.4280	0.4038	0.4280	0.4446	0.4408	0.4526
4	1	2	1	1	0.392	0.4144	0.3013	0.3073	0.3174	0.3181	0.3215
4	2	2	1	1	0.447	0.4669	0.3489	0.3562	0.3609	0.3650	0.3674
4	1	2	2	1	0.533	0.5401	0.5691	0.5607	0.5655	0.5666	0.5657
4	2	2	2	1	0.598	0.5834	0.6133	0.6047	0.6156	0.6153	0.6156
4	1	2	3	1	0.411	0.3964	0.3792	0.3964	0.4056	0.4087	0.4106
4	2	2	3	1	0.451	0.4430	0.4194	0.4430	0.4515	0.4541	0.4559
4	1	1	1	2	0.394	0.3587	0.3484	0.4124	0.3161	0.3992	0.3326
4	2	1	1	2	0.406	0.3674	0.2915	0.4197	0.3341	0.1099	0.3502
4	1	1	2	2	0.552	0.4927	0.5498	0.4927	0.5498	0.5455	0.8691
4	2	1	2	2	0.581	0.5184	0.5711	0.5184	0.5711	0.5663	0.5758
4	1	1	3	2	0.415	0.4240	0.3727	0.4639	0.3928	0.3702	0.4004
4	2	1	3	2	0.439	0.4313	0.3947	0.4700	0.4030	0.3890	0.4165
4	1	2	1	2	0.434	0.4489	0.3779	0.4811	0.3624	0.3774	0.4001
4	2	2	1	2	0.448	0.4618	0.4145	0.4923	0.3814	0.3153	0.4164
4	1	2	2	2	0.539	0.4966	0.5597	0.4956	0.6168	0.5662	0.6431
4	2	2	2	2	0.614	0.5201	0.5712	0.4110	0.6367	0.5874	0.6608
4	1	2	3	2	0.458	0.4840	0.4176	0.5124	0.4159	0.3458	0.4473
4	2	2	3	2	0.458	0.4964	0.4278	0.5233	0.4339	0.3669	0.4631

Composition of basal media.

Difco medium.

Bacto-asparagine
L-histidine hydrochloride
DL-methionine
DL-tryptophane
DL-isoleucine
DL-valine
Bacto-dextrose
Thiamine hydrochloride
Calcium pantothenate
Nicotinic acid
Biotin salt
Riboflavin

Inositol
Boric acid
Monopotassium phosphate
Magnesium sulfate
Ammonium sulfate
Calcium chloride
Potassium iodide
Ammonium molybdate
Manganese sulfate
Copper sulfate
Zinc sulfate
Ferrous sulfate

Standard medium

Thiamine
Biotin
Niacin
Magnesium sulfate
Manganese sulfate monohydrate
Potassium phosphate monobasic
Polysorbate 80 (Tween 80)
Potassium citrate
Casamino acids (Difco)
DL-tryptophan
DL-methionine
DL-valine

Inositol
Calcium pantothenate
Potassium Chloride
Ferric chloride
Calcium chloride dihydrate
Ammonium phosphate dibasic
Ammonium phosphate
Citric acid
Dextrose
L-histidine
DL-isoleucine

MICROBIOLOGICAL ASSAY VARIABLES FOR DETERMINING VITAMIN B-6 CONTENT OF CHICKEN MUSCLE

by

PAULA FELDER MARMET

B. S., Kansas State University, 1976

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Foods and Nutrition

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1986

ABSTRACT

Methodology variables in the microbiological assay for determination of vitamin B-6 content of chicken muscle were tested. Those included length of time for incubation, type of media, composition of standard curve and method of constructing standard curves for calculating vitamin B-6 content. Values interpolated from standard curves containing 80% pyridoxamine, 10% pyridoxal and 10% pyridoxine were significantly higher ($P<.05$) than those interpolated from standard curves containing equal amounts of the three forms of vitamin B-6 or from those containing pyridoxine only.

Extending the estimated optimum incubation time by two hours did not significantly affect determined B-6 values. Estimated optimum incubation time when using Difco media was determined to be 26 hr; estimated optimum incubation time when using the standard AOAC medium was determined to be 22 hr when standard solution concentrations were 1 ng/ml. Determined vitamin B-6 values of assays using the standard AOAC medium were higher than those determined from assays using the prepared Difco medium. Difference was significant at the 10% level for the "hand" method of calculation.

Vitamin B-6 values determined from standard curves which were computer calculated from linear or quadratic equations were generally lower than those which were determined from a standard curve drawn by "hand"; however, the difference was not significant except when the standard curve contained PN

only.

Observed results of column separation of the individual forms of the vitamin indicate that the tissue content of vitamin B-6 in cooked chicken muscle is approximately 70% PM, 20% PN and 10% PL.