Computer Programs for the Evaluation of Vitamin B Data Obtained by Microbiological Methods

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A four-part computer software package has been developed for evaluating microbiological vitamin assay data. The first unit plots the dosage–response curves with coded lines and includes legends and axes labels. The second unit evaluates standard and test dosage–response curves for parallelism since parallelism is a criterion of validity. If the curves are parallel, the standard and sample data are pooled to give a calibration curve from the larger population. Test responses deviating from parallelism due to stimulation and/or inhibition are identified. Vitamin contents of the samples are calculated, along with the percent standard error and confidence limits at the 5% level. Data from a survey of the vitamin contents of wine were evaluated by this program and compared to the results obtained by manual calculations. The third unit incorporates a statistical technique for determining the minimal and maximal concentrations of vitamins that can be assayed microbiologically. A portion of this program determines when the growth of microbial vitamin assays has stabilized. The last software unit evaluates the effects of inhibitors and/or stimulants on vitamin assays.

Vitamin assays depend on the relationship between the vitamin dosage and the test organism response. As the dose is increased from that which gives no effect to one giving a maximum effect, the response increases continuously as a sigmoidal dosage–response curve. Statistical techniques for handling sigmoidal curves are not as simple as those for evaluating linear data. In consequence, an initial step is to select units of response and of dose that will plot as a straight line over a range wide enough for assay purposes. Response is plotted on the ordinate against the logarithm of the dose on the abscissa. Since the dosage–response data are not linear, the logit transformation of the response is made (Ashton, 1972). The next step is to determine the straight line equation that best fits the dosage–response relationship. This can be computed by applying the principle of least squares. The equation of the line is known as the regression equation and its slope and intercept as the regression coefficients. It is then possible to determine whether the sample potency is independent of the level of dosage. That is when the response is plotted as a function of ln dose, the resulting sample and standard curves must be superimposable by horizontal shifting. This is equivalent to determining whether the dosage–response curves for the standard and the unknown are parallel within the sampling error. The sampling error is computed from the variation in the assay response. With two curves, the validity of measuring the unknown in terms of the standard can be tested before computing the relative potency. If the standard and unknown curves are similar, the data can be pooled to obtain a more reliable estimate of the slope from the larger data population than is available from the data of either curve alone. The variability of the observations around their respective curves will usually agree within the experimental error. This condition may usually be assumed, but if necessary, conditions defining an outlier can be generated in order to exclude outliers (Schatzki and Keagy, 1975). The combined slope of the two lines is determined from the numerators and the denominators of the individual slopes of the standard and the unknown. The next step is to estimate the sample potency. This is determined from the horizontal distance between the curves. The horizontal distance is in log concentration which can be converted to concentration by taking the antilogue. Multiplication of these values by the sample dilution factors gives the vitamin content per gram or milliliter of the original sample. The unknown response curve should explain at least 90% of the variability of the unknown dosages (Bliss and White, 1967). The minimum of three sample dilutions (preferably five) should agree within ±10% of their common mean (Strohecker and Henning, 1966). If these conditions are not fulfilled, the determination is not valid. The coefficients obtained from the parallelism test are then evaluated. If the slopes differ significantly, the direction of the divergence of the unknown with respect to the reference is determined. An unknown with a greater slope than the standard contains stimulants, while a lesser slope indicates the presence of inhibitors. The phenomenon is known as "drift" (Association of Vitamin Chemists, 1966). If a significant level of drift is present, the analysis is not valid. The interfering substances must be removed, and the purified extract analyzed; or possibly, a different test organism should be used (Pearson, 1967; Voigt and Eitenmiller, 1978).

The programs presented in this paper were developed in order to minimize the time required to evaluate data obtained from vitamin analyses. The statistical evaluations employed in these programs are too cumbersome to complete manually on a routine basis. Brolund et al. (1973) have developed a computer program to evaluate vitamin assay results. Their program does not evaluate the sample response data for drift and does not statistically evaluate the data.

METHODS

A. Plotting and Labeling Graphs. An example illustration from the plotting and labeling programs (Programs 1 and 2; see Supplementary Material Available paragraph) is Figure 1. The programs are written for the Hewlett-Packard 9820 A equipped calculator with a plotter.

B. Analysis of the Vitamin Contents of Food and Wine. Programs 3, 4, and 5 (Supplementary Material Available paragraph) were developed to evaluate vitamin content data. Programs 3 and 4 were written for the IBM 370, while program 5 uses the Cyber 74. The vitamin B contents in food and wine were determined using microbiological methods of vitamin analysis (Voigt et al., 1978a,b). Four aliquots (0.25, 0.5, 1.0, and 1.5 mL) of vitamin extracts from wine were analyzed.

Sigmoidal dosage–response calibration and test curves were transformed by the logistic function into linear curves. The transformation is illustrated in Figure 1. The trans-

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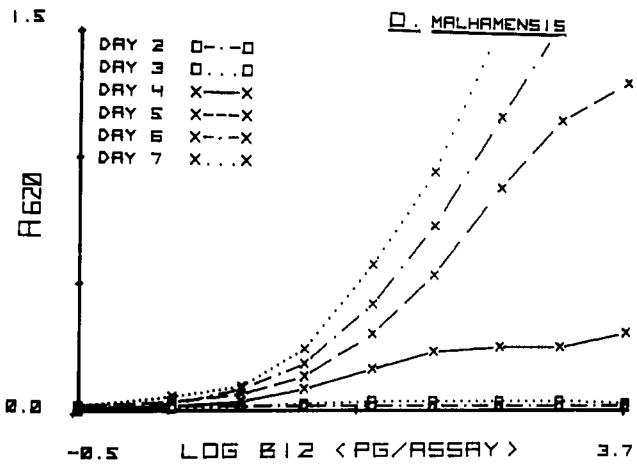

Figure 1. Example of a figure plotted and labeled by Programs 1 and 2.

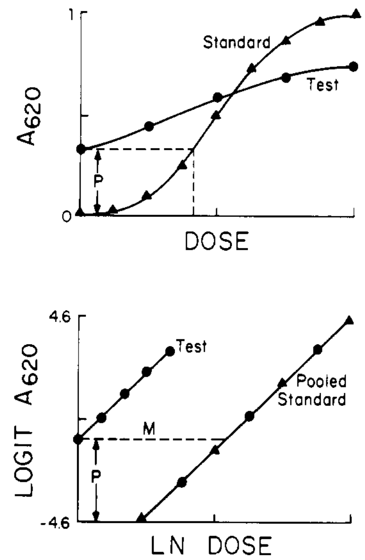

Figure 2. Dosage–response curves for the test and standard vitamin assays. Dose means "aliquot added" for the test or "vitamin concentration" for the standard. "P" is the response of the smallest test aliquot. (Top) Dosage–response curves that have not undergone transformation. The x axis has two scales (milliliter and nanogram). (Bottom) Transformed dosage–response curves. "M" is the ln dose ratio. The exponential of "M" is the vitamin potency of the test. The x axis intercept of the pooled standard curve is the vitamin concentration at the lowest sensitivity. The x axis has one unitless scale.

formed data for the two curves plotted in the bottom graph of Figure 1 do not intersect since only one scale (unitless) is used for the x axis vs. two scales (milliliter and nanogram) for the x axis in the top graph of Figure 2. The validity of the test responses were evaluated by testing for parallelism of the test and standard responses; that is, the null hypothesis tested whether the difference between the two slopes equaled zero. The Student's t test was employed at the 5% probability level to evaluate the null hypothesis. The concentration of vitamin in the test was obtained by taking the exponential of the ln (dose ratio), "M" in Figure 2 or M = ln (test potency/standard potency) and multiplying it by its dilution factor. M is the horizontal difference between the test and standard lines and gives the difference in ln (dose) for equal responses and is called the ln (potency ratio). This value represents the geometric mean of a lognormal distribution and not the arithmetic mean of a normal distribution of the vitamin content. Percent standard errors for each analysis were calculated. Ideally, they should be less than 10%. Confidence limits at the 5% level were also calculated for the mean vitamin contents (Bliss and White, 1967). These limits are skewed due to the bias entered into the calculations by employing logarithms, which gave lognormal vs. normal distributions. Log bias can be compensated for by further calculations, but this correction is not customarily made (Beuchamp and Olson, 1973; Thoni, 1969). A sample calculation is given in Chart I.

The statistical formulas used in the calculations are as follows:

(1) $M = \overline{\ln \text{dose}_s} - \overline{\ln \text{aliquot}_t} + (\overline{\text{logit}_t} - \overline{\text{logit}_s})/b$

where s = standard, t = test; logit = ln $[A_{620}/(A_{620} - 1)]$; $b = \sum(xy)/\sum(x^2)$; $\sum(x^2) = \sum(x - \bar{x})^2$; $\sum(xy) = \sum(x - \bar{x})(y - \bar{y})$; exp M = concentration in test.

(2) Schatzki and Keagy (1975) variance for M:

$$\mathrm{V}(M) = (2E)/(2b^2t)(n_s + n_t - 3)$$

$$S_M = \sqrt{\mathrm{V}(M)}$$

2 where E = minimum least-square error, t = Student t at
13 5% level, n_s = number of tubes for standard, n_t = number
25 of tubes for test.

(3) $\%S_M$ potency = $100[\exp(S_m - 1)]$

(4) Approximate limits (low estimate of sampling slope error):

$$x_{1m} = M \pm ts_M$$

30 where t is usually at the 5% level.

Available paragraph) evaluated the data from an incubation time study (Voigt et al., 1978c). Program 6 calculated and compared coefficients of dosage–response curves (IBM 370). Program 7 calculated the minimal and maximal vitamin concentrations that can be assayed by a given vitamin analysis method (Cyber 74). Data obtained from these calculations can be used to determine optimal incubation times for microbiological assays of vitamins.

Calibration assays for the microbial and protozoan methods of analyzing for vitamin B were prepared in triplicate. Four sets of calibration assays were prepared for the methods using bacteria and yeast as the test organisms. The assay sensitivity limits were determined by evaluating the slope changes in the inflection regions of the response curves. The regression coefficients of the calibration curves from the separate incubation periods were evaluated to determine the optimal assay incubation times. Optimal assay vessel sizes were determined by evaluating both the optimal incubation times and sensitivity ranges.

The sigmoidal dosage response curves were transformed by the logistic function to obtain linear regression coefficients. The transformation is illustrated in Figure 3. The slope and intercept coefficients were evaluated to determine the incubation time required for the slopes and intercepts to stabilize; that is, to determine when there was a nonsignificant change in slope and intercept with respect to time. Thus, the null hypothesis tested whether the differences in the slope and intercept coefficients from succeeding time intervals equaled zero. In this study, coefficients from each day were compared to those from the last day of incubation; that is, to the fourth day in the assays using bacteria and yeast. The Student's t test was employed at the 5% level to evaluate the null hypothesis. Both the intercept and the slope coefficients from the succeeding days had to be the same for the growth responses to be considered stabilized. Lack of symmetry between the upper and lower halves of dosage–response curves can be a problem when employing a generalized

Chart I. Computer Calculations for Evaluating Vitamin Contents

data for example					
standard			unknown		
ng of thiamin	absorbance		dose, mL	absorbance	
0	0.005	0.000	0.25	0.155	0.155
1	0.065	0.055	0.5	0.290	0.280
2.5	0.150	0.170	0.75	0.370	0.370
5.0	0.320	0.320	1.0	0.430	0.420
7.5	0.390	0.420	1.25	0.550	0.500
10.0	0.440	0.460			
15.0	0.540	0.550			
20.0	0.600	0.590			
25.0	0.690	0.670			

I. GLM analysis (Program 3)

a. The following data was obtained from Figure 6 (GLM Printout). The measure of the goodness of fit for the linear model to the logit transformation of the data was 0.988 (R-Square), $P \leqslant 0.0001$ (PR > F). Thus, the linear model gave a good fit to the data.

b. Parallel test: Slope of the standard (LCONC*G) only differs by −0.01619 from the test slope (1.06946 + (−0.01619) = 1.05337). The probability that this difference equaled zero (PR > T) was 0.8107, greater than our test level of $P \leqslant 0.05$. Thus, the standard and test curves were parallel.

c. The following data was coded for analysis to be conducted by Program 5 (Part III):
slope of unknown (B1): 1.06946; difference in slopes of standard and unknown (B2): −0.01619; SSR = 12.2081; XMSE = 0.01208; DF = 22; and DIL = 50.

II. Mean and CSS analysis (Program 4)

a. These computations yielded more data than was required for the calculations conducted in Part III. This information was punched onto cards, as well as being printed out. The data for this example was:

VIT = 1 MET = 1 RUN = 1 TYPE = 1 (std)
variable N Mean corrected SS
LWINE 16 −0.55441
WCONC 16 1.97073 16.78074
VIT = 1 MET = 1 RUN = 1 TYPE = 2 (unknown)
LWINE 10 −0.67001
WCONC 10 −0.42880 3.23098

or:

std:DN1 = 16; SS1 = 16.78074; CONC1 = 1.97073; STD = 0.56926
unknown:DN2 = 10; SS2 = 3.23098; CONC2 = 0.42880; TEST = −0.67001

III. Calculation of vitamin levels and sorting (Program 5)

a.

calculation		example data
B = ((2*B1) + B2)/2		= 1.061365
M = CONC1 − CONC2 + (TEST − STD)/B		= 2.290613
T1 = 2*XMSE*DF		= 0.53152
T2 = DNI + DN2 − 3		= 23
V = T1/2*B*B*DN2*T2		= 0.00102572
SM = SQRT(V)	= x(5)	= 0.032026
TT = TTP (.975, DF)		= 2.074
TS = TT*SM	= x(4)	= 0.066423
TE = 100*(EXP(SM)-1)	= x(6)	= 3.2544
B1 = M-TS		= 2.22418
B2 = M+TS		= 2.35703
X(1) = EXP(M)*DIL		= 494 ng/mL
X(2) = EXP(B1)*DIL		= 462 ng/mL
X(3) = EXP(B2)*DIL		= 528 ng/mL
X(7) = 0.80*X(1)		= 395 ng/mL
X(8) = 1.2*X(1)		= 593 ng/mL

b. Sorting: The sorting section of the program grouped the data (rows) from a given vitamin together (e.g., thiamin) which was subgrouped by: (1) all data derived from the same method of analysis (e.g., *L. viridescens*); (2) by data from consecutive runs; and (3) into a consecutive listing of data from the same sample type. The columns on the printout represent the vitamin abbreviation, analysis method number, run number, sample number, mean vitamin content, lower limit of mean at 95% level, upper limit of the mean at 95% level, standard error at 95% level, standard error, percent standard error, and mean vitamin content plus and minus 20%.

formula for calculating the sensitivity limits. A sample calculation is given in Chart II. The slope and intercept coefficients for any given day were substituted into the following formula to determine the sensitivity limits at any given rate of change in slope:

$$A = 0.5 \pm \sqrt{(b - 4D)/4b} \tag{5}$$

$$\text{dose} = b\sqrt{A/(1 - A)e^{a}}$$

$$D = (b - 4bx^2)/4$$

where x = estimated $A - 0.5$, b = slope, a = intercept, D = rate of slope change, A = absorbance at the calculated limits, dose = concentration at the calculated limits.

D. Effect of Chemical Additives. Programs 8 and 9 (IBM 370 and Cyber 74, respectively; see Supplementary Material Available paragraph) calculated the data from a study on the effect of the addition of chemicals to microbial vitamin assays (Voigt et al., 1978d). Program 8 calculated the quadratic coefficients, while Program 9 calculated the chemical concentrations that correspond to certain points of interest, e.g., the no effect level, stimulation limits and 50% inhibition. These programs may be used to select acids and bases to use for vitamin extractions and media pH adjustments.

The effect of food preservatives and neutralization salts

Chart II. Computer Program for the Selection of Optimal Length of Incubation and Calculation of Minimal and Maximal Vitamin Concentrations (Assay Volume = 5 mL)

data for example

vitamin B_{12} (pg/assay)	absorbance											
	day 1			day 2			day 3			day 4		
0	0.020	0.015	0.015	0.030	0.030	0.030	0.055	0.065	0.055	0.050	0.045	0.055
25	0.150	0.150	0.130	0.220	0.220	0.235	0.290	0.270	0.260	0.265	0.280	0.290
50	0.240	0.250	0.240	0.420	0.420	0.430	0.510	0.490	0.490	0.470	0.570	0.470
75	0.295	0.310	0.300	0.590	0.630	0.560	0.620	0.610	0.630	0.710	0.650	0.650
100	0.340	0.360	0.350	0.630	0.660	0.680	0.670	0.660	0.640	0.740	0.760	0.760
125	0.385	0.390	0.390	0.740	0.740	0.710	0.820	0.820	0.830	0.780	0.780	0.800
150	0.430	0.390	0.410	0.800	0.790	0.770	0.870	0.850	0.860	0.850	0.830	0.860
250	0.550	0.540	0.520	0.850	0.860	0.850	0.880	0.900	0.920	0.920	0.930	0.920
500	0.660	0.650	0.650	1.000	1.020	1.020	1.000	0.950	1.050	1.080	1.100	1.100
1000	0.740	0.70	0.750	1.500	1.500	1.150	1.180	1.100	1.200	1.200	1.250	1.250

I. GLM analysis for optimal incubation time (Program 6)

a. Figure 7 is a printout from the data given above. The study was the effect of up to 4 days of incubation on the *L. leichmanii* assay for vitamin B_{12}. The top half of the column in the printout entitled, "ESTIMATE" lists the intercepts while the bottom half lists the slope coefficients corresponding to each day's dosage-response curve. The first intercept or slope value corresponds to the coefficients from the fourth day. The coefficients that follow these values correspond to the difference between the coefficients of the day specified vs. the fourth day. The null hypothesis tested whether these differences were significantly different from zero. Both the intercept and slope coefficients had to significantly equal zero ($P \leqslant 0.05$) for the incubation period to be confirmed as yielding a stable dosage-response curve.

b. The following data was derived from this analysis for substitution into Program 7, which calculated the sensitivity limits (the notation used in Program 7 is indicated in parenthesis). Final day intercept (A1): −5.77533; stable day intercept (A2): −0.65473; final day slope (B1): 1.49118; stable day intercept (B2): 0.06865.

II. Calculation of sensitivity limits (Program 7)

a. All data were entered manually as requested by the printed display of the computer terminal. The intercepts and slopes from the dosage-response curves of the longest incubation period and from the earliest stable incubation period were entered (A1, A2, B1, B2). Estimates of the absorbance limits within which the upper assay absorbance was expected to occur could be entered or the values 0.5 and 0.99 could be entered (OD1, OD2). When the latter are entered, the program calculates all the concentrations corresponding to each unit of percent change in slope between 0.5 and 0.99, along with the corresponding lower concentrations (0.01 to 0.5). The program assumes the dosage-response curve is symmetrical.

b. Example calculations (ALOG = natural logarithm not A*LOG)

calculation	example data
OD1 = OD1 −.5	= 0
OD2 = OD2 −.5	= .49
A = A1 + A2	= −6.43006
B = B1 + B2	= 1.55983
Z1 = B-4*B*(OD1**2)	= 1.55983
Z1 = Z1/4	= 0.38996
Z2 = B-4*B*(OD2**2)	= 0.06177
Z2 = Z2/4	= 0.01544
K1 = Z2*100 } gives range of percent slope change values to be evaluated,	= 1.544
K2 = Z1*100 } this example will only use the value I = 5	= 38.996
D = I/100	= 0.05
TEMP = (B-4*D)/(4*B)	= 0.21795
TEMP = SQRT(TEMP)	= 0.4668
Y1 = 0.5 + TEMP	= 0.9668
Y2 = 0.5 − TEMP	= 0.0332
*Z2 = ALOG(Y2/(1-Y2))	= 3.37144
*Z1 = ALOG(Y1/(1-Y1))	= −3.37144
X1 = EXP((Z1-A)/B)	7.11 pg/assay
X2 = EXP((Z2-A)/B)	536 pg/assay

Assay volume was 5 mL thus the sensitivity would be 7.11/5 and 536/5 (X1 and X2) or approximately 1-100 pg/mL, the absorbance values that correspond to these limits were 0.03 and 0.97 (Y2 and Y1), the slope and intercept of the curve were 1.56 and −6.43 (B and A). The percent slope change was 0.05 at both limits. The choice of 0.05 (I = 5) was made after inspecting the concentration limit prophiles generated when the program scanned the absorbance range 0.5 to 0.99. When a large change in the indicated vitamin sensitivity occurred, the percent slope change corresponding to it was selected as the limit.

c. Format of output on the printout (left to right): Percent slope change, intercept, slope, minimal absorbance, maximal absorbance, minimal concentration and maximal concentration.

on microbial and protozoan assays for vitamin B were examined. The chemical concentrations studied and the assay conditions were described previously (Voigt et al., 1978d). Separate series of five aliquots (0.25, 0.5, 0.75, 1, and 1.25 mL) of the chemical solutions were added in duplicate to vitamin assays. Additive solutions of decreasing concentration were used until there was no measurable difference between a blank (water) addition and the chemical addition.

Figure 4 illustrates a theoretical dosage–response curve. The initial addition of low levels of the chemical produced stimulation, which was followed by inhibition at higher additive levels. Specific parameters describing this response are calculated: (1) the chemical concentration limits for stimulation ("UL_1" to "UL_2"), (2) the chemical concentration at maximal stimulation ("MAX") and its corresponding increase in absorbance ("ΔA_{620}"), (3) the maximal chemical concentration that had no detectable effect ("LL" or "UL_1" if stimulation was present), and (4) the chemical concentrations that inhibited the growth 50%

Chart III. Computer Calculations for the Effect of Chemical Additives on Vitamin Assays

data for example					
benzoate, mM	absorbance		niacin, ng/assay	absorbance	
0.07	0.710	0.710	500	0.690	0.690
0.14	0.730	0.750	1500, control	0.700	0.710
0.21	0.750	0.720	5000	0.720	0.730
0.28	0.720	0.730			
0.35	0.710	0.710			
0.7	0.720	0.690			
1.4	0.690	0.640			
2.1	0.570	0.530			
2.8	0.075	0.120			
3.5	0.035	0.030			

I. Quadratic model analysis (Program 8)

Figure 8 is a printout from the data given above. The data were from a study of the effect of increasing levels of sodium benzoate on the *T. pyriformis* assay for niacin. The column in the printout entitled "B VALUES" lists the quadratic coefficients corresponding to this dosage-response curve. The "R-square" value of 0.83 ($P \leqslant 0.001$) indicated a reasonable fit of the quadratic function to this data ["R-square" = (correlation coefficient)^2]. The coefficients to be used for further data analysis were as follows (the notation used in Program 9 is indicated in parenthesis): 0.60976 (cc); −0.266079 (B); −0.09616 (A).

II. Calculation of chemical concentrations (Program 9)

a. All data were entered manually as requested by the printed display of the computer terminal. Variation in the response measured when no chemical had been added to the assay had to be defined. This could have been done by either using a sufficient number of samples to calculate confidence limits using a *t* test (say ten samples) or one could estimate the limits. In this study, the limits were estimated by calculating the minimal and maximal absorbances that corresponded to a 15% change in vitamin concentration between the control level and the next higher and lower levels used in the standard curve (i.e., lower limit (A1), control (A2), upper limit (A3), lower absorbance (B1), control absorbance (B2), upper absorbance (B3), alpha-numeric code in parentheses was used in Program 9).

b. Example calculations

calculation	example data
SPU = B2 + 0.15*A2*(B3 − B2)/(A3 − A2), K = 1	0.70789
SPL = B2 − 0.15*A2*(B2 − B1)/(A3 − A2), K = 2	0.68138
SPH = B2*0.5 K = 3	0.3525
K = 1, C = CC − (K = 1)	−0.09813
DISC + B*B-4*A*C	0.03305
X1 = (−B− SQRT(DISC))/2/A	−0.43783
X2 = (−B+SQRT(DISC))/2/A	−2.32839
X1 = EXP(X1) } Intersection of upper limit of control	0.645
X2 = EXP(X2) }	0.0975
K = 2, C = CC − (K = 2)	−0.07162
DISC = B*B-4*A*C	0.04321
X1 = (−B− SQRT(DISC))/2/A	−0.30228
X2 = (−B+SQRT(DISC))/2/A	−2.46397
X1 = ESP(X1) } Intersection of lower limit of control	0.739
X2 = ESP(X2) }	0.085
K = 3, C = CC − (K = 3)	0.25726
DISC = B*B-4*A*C	0.16971
X1 = (−B− SQRT(DISC))/2/A	0.75892
X2 = (−B+SQRT(DISC))/2/A	−3.5252
X1 = ESP(X1) } Intersection at 50% inhibition level	2.136
X2 = ESP(X2) }	0.029
DER = −B/2/A	−1.383
XP = EXP(DER), concentration maximum stimulation	0.251 mM
FF = A*DER*DER+B*DER+CC, absorbance at maximum stimulation	0.794 absorbance

The two values derived from $K = 1$ (i.e., "Intersects upper spike at MM" on printout) define the concentration limits for stimulation, the second value (X2) defined the "no effect" concentrations when stimulation was detected. If only inhibition was detected, then the first value (X1) from the K = 2 calculation (i.e., "Intersects lower spike at MM" on printout) defined the "no effect" concentration. The concentration at 50% inhibition was defined by the first value (X1) from the K = 3 calculation (i.e., "Intersects half-spike at MM" on printout). The concentration at maximal absorbance (i.e., "Derivative is zero at MM" on printout) was given by XP, while the corresponding absorbance (i.e., "Value of at this point" on the printout) was given by FF.

("I_{50}"). These parameters were determined by fitting the quadratic function to the dosage–response data and then calculating the doses corresponding to the responses at these parameters. The response at "MAX" was evaluated by placing the first derivative of the quadratic equation equal to zero since the rate of slope change at this point would equal zero. A sample calculation is given in Chart III.

RESULTS AND DISCUSSION

The vitamin content of wine as calculated manually and by computer analysis is listed in Table I (Programs 3, 4, and 5). The occurrence of parallelism or nonparallelism of the sample curves and the standard curves are footnoted to the values in Table I. The percent standard errors (% SE) corresponding to these analyses are also given in Table I. The size of the percent standard error indicates the amount of scatter in the data. When all replicate runs showed deviation from parallelism, the run showing the smallest % SE was reported. Replications showing no significant deviation from parallelism were pooled, providing their % SE corresponded; otherwise, the value from the run having the smallest % SE was reported. Data from experiments showing nonparallelism are invalid. The data from the manual calculations did not include evaluations for parallelism or scatter. For the manual calculations, the

Table I. Vitamin Content of Wine as Calculated Manually and by Computer Analysis

wine	vitamin B_{12}, pg/mL[a] manual	computer[b]	% SE	niacin, ng/mL[a] manual	computer[b]	% SE
white wines						
Chenin Blanc	0.032	0.068	31	390n	380n	8
Sauvignon Blanc	0.007	NV[c,d]	1300[c]	640n	500n	11
Rhineskeller	0.002n	0.001n[c]	330[c]	1200n	950n	7
Rhine Castle	0.018	0.004[c]	50[c]	760n	630n	12
red wines						
Cabernet Sauvignon	0.053	0.11	29	440n	430n	8
Zinfandel	0.053	0.20	28	1200	800	7
Bergundy	0.070n	0.13n	34	1500	1100	8

[a] Values followed by "n" are not significantly different at the 5% level. [b] Pooled sample and standard curve slopes for the calibration curves had $R^2 \geq 0.9$ ($P \leq 0.001$). [c] Inhibition detected at the 5% level. [d] "NV" calculation not valid.

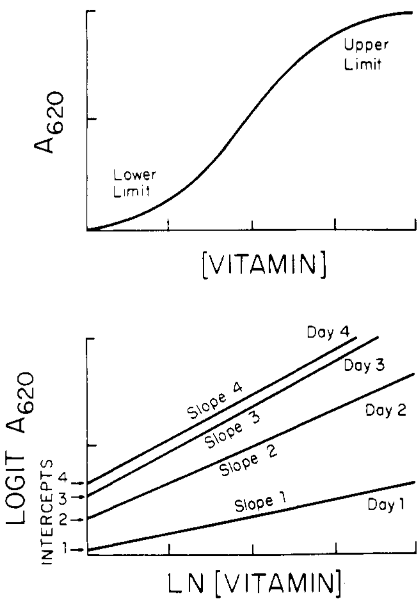

Figure 3. The effects of vitamin concentration, time, and assay vessel size on the growth responses of the vitamin assays were evaluated using the parameters in this figure. (Top) The lower and upper limits of the sigmoidal dosage–response curve. (Bottom) The intercepts and slopes of four transformed dosage–response curves. The four linear transformations correspond to four daily monitorings of a vitamin assay which was attaining stability in the measured growth response.

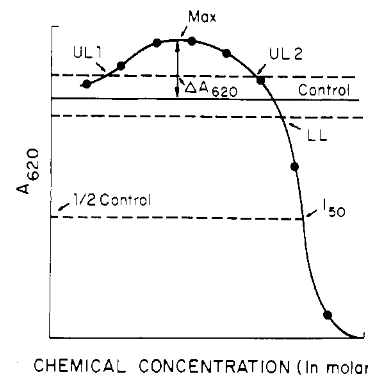

Figure 4. The inhibition and/or stimulation effects of the chemical additives on the vitamin assays were evaluated at the points indicated in this diagram. The solid line labeled "Control" is the measured growth in the vitamin assays containing no chemical additives. The confidence limits for the control are indicated by the dashed lines enclosing the "Control" line. "UL_1" and "UL_2" are the additive concentration limits for stimulation. "Max" is the additive concentration at maximum stimulation. "ΔA_{620}" is the measured absorbance increase at maximum stimulation. "LL" is the maximum additive concentration that had no effect; however, if stimulation was detected, the no effect level is defined by "UL_1". "I_{50}" is the additive concentration that inhibited the growth 50%.

absorbance of the growth of the test organisms were projected onto standard curves, multiplied by their corresponding dilution factors and averaged to determine their vitamin content. Nonsignificant differences at the 5% level between the values obtained by the computer analysis and the manual calculations are identified in Table I by following the vitamin values with the letter "n". Significant differences were obtained between the computer and manual calculations of the white wine vitamin B_{12} values. The significant differences were caused by the failure of the manual method to reject invalid (nonparallel) data. It is evident from the data in Table I that nonparallelism greatly increased the % SE.

The programs for calculating vitamin content yield more accurate data than the manual method and they easily handle the tedious calculations involved in evaluating the validity of experimental data. Validity evaluations are necessary to maintain credibility in the results from vitamin quantitations. The plotting programs decrease the time required to obtain graphics. The programs that evaluate sensitivity and the effects of chemical additives can be used in future studies to optimize vitamin extraction and assay methods.

The programs described use three computers. The Hewlett-Packard 9820A calculator with plotter is designed for desk-top use in a research facility. The IBM 370 is most efficiently used to evaluate complex calculations that require a large computer core space. The Cyber 74 efficiently evaluates less complex calculations, and data can be entered manually from remote computer terminals.

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Supplementary Material Available: Listings of computer programs, data input and output formats, and discussion of the operation of the programs are given (Programs 1–9; Tables II–XII and Figures 5–8; 31 pages). Ordering information is given on any current masthead page.

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Tin Binding in Canned Green Beans

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Tin distribution was studied in green beans from detinned cans and in tin-free green bean puree incubated, under nitrogen, with stannous citrate. Tin was determined by colorimetry of a phenylflurorone–Sn^{4+} complex. Canned beans were drained, homogenized, and centrifuged. Approximately 90% of the total tin remained in the drained beans. Ninety percent of this tin was recovered in the centrifugation sediment (up to 21 mg of tin/g dry weight) and could not be extracted from it by acid, alkaline, or saline solutions. Ethylenediaminetetraacetic (0.2 M) and 0.05 M cysteine solutions released respectively 39 and 30% of this bound tin. Pectinases plus cellulases, or α-amylase plus glucoamylase, released no tin; proteases released up to 13%. The model system yielded similar results. In both cases, stannous ions appear to be strongly bound to insoluble bean constituents otherwise than by electrostatic attraction or physical adsorption. Such bindings may account for the absence of toxicity of tin in solid canned foods.

The level of tin in vegetable and animal tissues, and therefore in most human foods, is generally less than 2 mg/kg (Schroeder et al., 1964). Canned foods, however, usually contain 10–100 mg of tin/kg and sometimes several hundred mg/kg (Kolb, 1975) as a result of the corrosion of the tin plate container. The tin dissolves as stannous ions (Sn^{2+}), and the corrosion depends on many factors (Kamm et al., 1961; Hoare et al., 1965; Willey, 1972).

Tin levels up to 200–250 mg/kg have come to be regarded as normal in canned foods (unlacquered cans), and the experience of the large consumption of canned foods shows indeed that the small amounts of tin which they add to the daily diet do not represent a risk. Schroeder et al. (1964) calculated that the daily intake of tin for an adult man in the United States is about 4 mg; the level in various organs was found to be less than 1 mg/kg. Tin does not accumulate in organs of rabbits or rats (Kutzner and Brod, 1971; Fritsch et al., 1977a,b). Absorption studies in man and animals have shown that most of the ingested tin is excreted in the feces (Calloway and McMullen, 1966; Benoy et al., 1971; Hiles, 1974; Fritsch et al., 1977a,b). Tin has been shown to be an essential element for the rat, at a level of 1–2 mg/kg of food (Schwartz et al., 1970, 1974).

The ingestion of liquids (fruit beverages, solutions of stannous chloride or citrate) containing high (250–2000 mg/L) levels of tin may, however, elicit acute temporary gastrointestinal troubles in man, monkey, dog, and cat (Calvery, 1942; Benoy et al., 1971; Cheftel and Truffert, 1972).

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Short-term toxicity studies (De Groot et al., 1973a,b,c; De Groot and Willems, 1974) have essentially shown that the toxicity of tin varies greatly with the solubility and is apparently related to the supply and metabolism of iron (De Groot et al., 1973c; Kappas and Maines, 1976). Long-term studies with $SnCl_2$ and Na_2SnCl_2 have shown no adverse effects in mice and only slight ones in rats (Roe et al., 1965; Schroeder and Balassa, 1967).

Little is known, however, regarding the chemical form in which stannous ions may be bound in certain foods and thereby become less toxic.

In fruit-based beverages packed in plain tinplate cans, 75–90% of the dissolved tin is present in soluble and dialyzable form, possibly as organic acid chelates (Sherlock and Britton, 1972; Willey, 1972; Albu-Yaron and Semel, 1976). In many solid or partly solid canned foods, including fruits and vegetables, most of the tin appears to be bound to insoluble constituents of the food (Heintze, 1959, 1960; Horio et al., 1966, 1970, 1972; Woidich and Pfannhauser, 1973). Proteins (Gruenwedel and Hao, 1973), polyphenols (Heintze, 1959, 1960), and perhaps chlorophyll and pectins, are able to complex stannous ions in the form of insoluble or soluble chelates.

In order to investigate these points, we have studied the distribution of tin in canned green beans, as well as in a model system (green bean puree incubated with stannous citrate), and the capacity of some fractions of the material to bind stannous tin and to retain it when submitted to extraction with various solvents. Model system studies are difficult because: (a) stannous ions, which remain as Sn^{2+} in the anaerobic conditions of the food in the sealed can, quickly oxidize into Sn^{4+} in the presence of oxygen; (b) both stannous and stannic ions, in an aqueous solution free of complexing agents, between pH 2 and 11 give colloidal

