EVALUATION OF METHODS TO DETERMINE THE SULFUR CONTAINING AMINO ACIDS IN POTATOES

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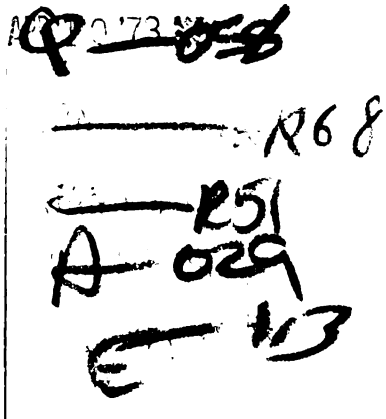

ABSTRACT

EVALUATION OF METHODS TO DETERMINE
THE SULFUR CONTAINING AMINO ACIDS IN POTATOES

By

Robert Luescher

The methionine content of six potato varieties were estimated by Leuconostoc mesenteroides P-60 with acid hydrolysis and by Streptococcus zymogenes using both acid and enzymatic hydrolysis techniques. By applying the same assay structure, the three methods could be compared for accuracy, recovery and economics. All methods differentiated high, medium, and low methionine contents. Values received from L. mesenteroides were approximately 30% lower than those obtained by S. zymogenes and acid hydrolysis. With two exceptions the latter ones agreed with the results obtained through ion exchange chromatography (Kaldy, 1971).

The technique using S. zymogenes and enzymatic digestion was found to be most appropriate for analyses of a large number of samples since the procedure was least time consuming and the results accurate enough to differentiate among high, medium and low methionine contents. This method permits measurement of the "available" methionine which may correspond more closely with the results in "vivo."

Cystine analysis for the same cultivars was conducted with L. mesenteroides. Values obtained were about half of those from ion exchange chromatography. A definite explanation of this phenomena could not be given.

In further assays ten randomly chosen cultivars from each of 18 families were analyzed for methionine using S. zymogenes and enzymatic hydrolysis. From the variance of the population mean, 43% could be explained by differences within families, 55% by differences among families and 2% by variance due to the method. Methionine varied over a range of 1.0 up to 3.6 µg l-met per mg d.m.

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By

Robert Luescher

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INTRODUCTION

Among the world's most important food crops, the potato ranks first in terms of volume of fresh product. While usually classed as a carbohydrate, it has been in most cases by-passed as a source of protein. In its fresh state, it has only an average of 2% "total protein." However, on a dry basis the "total protein" content of potatoes is not different from that of wheat. Also, an important fact overlooked by many is that one hectare of land under potato cultivation can supply the protein requirement for 9.5 people, while the protein of wheat from the same land can satisfy only 6.3 people (Borgstrom, 1969). Moreover, the biological value for the potato for the human adult is 72 compared to 53 for wheat flour (FAO, 1957).

Investigations have shown (Watts et al., 1959; Williams, 1959) that the delivery of methionine for human nutrition is a pathway and it appears that this amino acid limits the biological value of potato protein (Williams, 1959; Schuhpan, 1958; Rios, 1969).

Therefore, it would be very desirable to develop a potato with an increased methionine content. To achieve this aim, the potato breeder needs simple methods to screen large numbers of potato clones for methionine.

The purpose of this thesis is to find and adapt an appropriate method for the above mentioned purpose. This method must be fairly accurate, rapid, and economical. Since cystine may substitute in the diet for about 30% of the methionine (Albanese and Orto, 1968), an appropriate method for the analysis of cystine would be desirable.

LITERATURE REVIEW

Method to determine methionine

There are good methods for the determination of some amino acids, for instance tryptophan (Spies and Chambers, 1948; Spies, 1967). The sulfur containing amino acids are the most difficult to determine. Both methionine and cystine are very unstable during acid hydrolysis, especially in the presence of carbohydrates (Block, 1956 a). At present there are no rapid or simple methods available for determining methionine and cystine.

Advantages and disadvantages of ion exchange chromatography

The introduction of an automatic chromatographic separation procedure for amino acids (Spackman *et al.*, 1956) opened a new area in this field. Spackman *et al.* (1956) reported an accuracy of 100 ± 3% and in less than ten hours an analysis of almost all amino acids could be performed.

Some difficulties were reported in the recovery of methionine and cystine (Block, 1956 a). The same problem was observed when the same samples were analyzed by Kaldy (1971) and in this study.

A special oxidation of cystine and cysteine to cysteic acid and methionine (Schram et al., 1954; Lewis, 1966) to methionine sulfone was necessary in order to get satisfying results for these amino acids.

Furthermore the amino acid tryptophan must still be determined in a separate analysis by a special method (Spies and Chambers, 1948; Spies, 1967).

Since the protein has to be broken down to its amino acids by means of acid or alkaline hydrolysis, the "total" amino acids are determined by chromatographic methods.

Furthermore the cost for a total analysis is too high to be practical for screening segregating populations in a heterozygous tetraploid.

Advantages and disadvantages of paper chromatography

Since paper chromatography requires simple equipment, this procedure is much less expensive than ion exchange chromatography. But the quantitative determination of single amino acids is quite laborious and the accuracy is only about 100 ± 6% (Mulder and Bakema, 1956). Furthermore acid hydrolysis and the removal of the acid (HCl) is required for both paper chromatography (Mulder and Bakema, 1956) and for ion exchange chromatography (Spackman et al., 1956).

Advantages and disadvantages of microbiological methods

All microbiological assay methods in current use are similar and can be applied with equal facility to any amino

acid for which techniques have been established. The value, especially for routine analysis, is readily apparent (Block, 1956 b). The growth of living cells is subject to many more extraneous influences than are in-vitro chemical reactions or physical measurements (Kidder and Dervey, 1949). The most important advantages of microbioassays for amino acid analyses are that there are highly specific bacteria and simple techniques applicable for all amino acids. The methods are well adapted to routine work (Guggenheim, 1970).

Choice of appropriate microorganisms

Only L. mesenteroides and S. zymogenes can be used for methionine and cystine analyses (Table 1).

Table 1: Bacteria and the amino acids for which they have a specific requirement.

L. arabinosus	leucine, isoleucine, valine, tryptophan, glutamic acid
L. helveticus	serine, aspartic acid
L. brevis	glycine, proline
L. mesenteroides	lysine, phenylalanine, histidine, serine, glycine, proline, aspartic acid, methionine, cystine, glutamic acid, tyrosine, arginine, leucine, isoleucine, valine
S. faecalis	lysine, hystidine, arginine, threonine
S. zymogenes	methionine, leucine, isoleucine, valine, arginine, histidine, tryptophan, glutamic acid
Pediococcus cerevisiae	alpha-alanine

Source: Based on Barton-Wright (1952) and the newer studies of Ford (1962).

MATERIALS AND METHODS

I. Methods and characteristics of S. zymogenes

The standardized method used in these tests is a modification of Boyne et al. (1967).

Reagents

Assay medium: The assay medium was prepared from two stock solutions, the basal medium and the amino acid supplement, made up as described in Appendix 1 and 2.

Stock solutions were stored at -20 C. When required for use, the stock solutions were thawed and the amino acid supplement warmed to dissolve the precipitate. They were then combined in the proportion of two volumes of basal medium to one volume of amino acid supplement.

Organism maintenance and inocula

Streptococcus zymogenes (NCDO 592) was obtained from the National Culture of Daily Organisms of the National Institute for Research in Dairying.

Stock cultures were maintained by weekly (1) transfer (on Bacto Assay Culture Agar B 319, Difco Laboratories, Inc., Detroit, Mich.), incubation over night, and storage at 2 C (2). To prepare the inoculum for the assay, the

broth (Bacto Micro Inoculum Broth B 320, Difco Laboratories, Inc., Detroit, Mich.) was inoculated directly from the stock culture (3) and incubated over night at 37 C.

Modifications: (1) weekly transfer instead of monthly transfer
(2) 2 C instead of 4 C
(3) transfer medium was omitted

a. Procedure for "available" methionine

The "available" methionine content was determined by using S. zymogenes and enzymatic hydrolysis. For "total" methionine, acid hydrolysis was used instead of enzymatic hydrolysis.

Preparation of the samples for tests: The freeze-dried samples (preparation see page 14) were ground to pass an 80 (1) mesh sieve. A half gram (2) ± 0.5% was weighed into 4 oz. wide mouth screw cap bottles (in duplicate) and suspended in 20 ml of citrate cyanide buffer (see Appendix 3). The pH was adjusted to 7.2 and the container placed in a water bath at 56 C. Two ml of 4% (W/V) crude papain (Difco Laboratories, Inc., Detroit, Mich.) in citrate cyanide buffer at pH 7.2 was added and incubated with intermittent shaking for 3 hr at 56 C. The pH of the digest was adjusted to 7.2. Then, 78 ml of distilled water was added bringing the volume to 100 ml ± 0.5 ml.

Determination of "available" methionine: Triplicate portions of 0, 1, 2, 3, 4, 5, 6, 7, and 8 ml of the standard methionine (10 µg l-met/ml) were distributed into 16 x 150 mm test tubes. Duplicate 2, 3, and 4 ml portions of each sample were pipetted into the same sized test tubes. Three ml of assay medium was added to each tube and brought to the final volume of 11 ml with distilled water. Each tube was covered with a test tube cap, sterilized by steaming (100 C) for 20 minutes, and cooled in a water bath to 37 C. Then one drop of inoculum culture that had been diluted 1:10 with 0.85% saline solution (3) was added and the samples incubated at 37 C for 48 hours.

Modifications: (1) 80 mesh instead of 40 mesh
(2) 0.5 g instead of 100 mg N
(3) undiluted inoculum was used in the original paper

In this case a sample of 0.5 g potato starch was used as a blank.

Measurement of growth response: After incubation the tubes were heated in flowing steam for 10 minutes and cooled to room temperature. The tubes were stoppered and shaken very vigorously, then set aside for 30 seconds to allow air bubbles to rise but not food particles to settle. Correction of turbidity caused by food particles is outlined on page 26. The optical densities of the cultures were

measured with a Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer with a flow through cell at 580 μm (Ford, 1960).

Calculation of results: A curve of the average responses of the standard cultures was plotted. From this curve the values for each sample culture were calculated. Samples with cultures which differed more than 15% of its mean were repeated.

Characteristics of S. zymogenes

S. zymogenes has an absolute requirement for exogenous leucine*, methionine*, tryptophan*, arginine, histidine, isoleucine*, valine*, and glutamic acid. Of the "essential" amino acids, lysine, threonine, and phenylalanine were not absolutely essential to this bacteria though the omission of any one from the culture medium caused a marked drop in growth rate (Ford, 1960).

Furthermore Ford (1960) describes S. zymogenes as follows: "It requires much the same pattern of exogenous amino acids as Tetrahymena: it is powerfully proteolytic, and grows quickly with an adequate intact protein as the main source of nitrogen."

Its ability to work as a proteolytic microorganism makes S. zymogenes a very important tool. Instead of chemical hydrolysis, the enzymatic predigestion is

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*Essential for man.

satisfactory. Chemical hydrolysis may cause destruction or unavailability of certain amino acids in the food protein, especially when large amounts of carbohydrates are present (Evans and Butts, 1948; Block, 1956 a). Acid hydrolysis can also destroy growth inhibitory substances. Enzymatic digestion permits measurement of the "available" amino acids which may more closely correspond with the results in "vivo."

The saving of time by enzymatic digestion compared to the acid hydrolysis is shown later. Unfortunately it is not possible to determine "available" cystine from prepared enzymatic hydrolysates. In this respect L. mesenteroides is a superior microorganism because "total" cystine as well as "total" methionine can be determined from the same acid hydrolysate.

b. Procedure for "total" methionine

The methionine analyses follow similar procedures except for preparation of the samples for analysis: Duplicate 0.5 g samples were weighed into 50 ml Erlenmeyer flasks and hydrolyzed as described by Evans et al. (1962): Ten ml of 20% HCl was added to each flask. After autoclaving for 30 minutes at 15 pounds pressure (121 C) the samples were cooled in ice water, the pH adjusted to 7.0 with 12% and 4% NaOH.

Then the samples were filtered and brought to a volume of 100 ml with distilled water.

II. Method using Leuconostoc mesenteroides P-60 for "total" methionine

The standardized method was based on that described in the Difco Manual (1969) using dehydrated Bacto-Methionine Assay Medium (Difco Laboratories, Inc., Detroit, Mich.) according to the formula given by Steel et al. (1949).

Reagents

To rehydrate the medium, 105 grams of Bacto Methionine Assay Medium was suspended in 100 ml distilled water and filtered before use.

Organism maintenance and inocula

L. mesenteroides (ATC 8042) cultures were maintained and test samples prepared as described under S. zymogenes (pages 7, 11). Triplicate portions of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 ml of the standard methionine (15 µg l-met/ml) were distributed into 18 x 150 mm test tubes. Duplicate 0.4, 0.8, and 1.2 ml portions of each sample were pipetted into the same sized test tubes. Five ml of the prepared Bacto-Methionine Assay Medium was added to each tube and brought to the final volume of 10 ml with distilled water. Each tube was covered with a test tube cap, sterilized in an autoclave at 15 pounds pressure (121 C) for 10 minutes, and cooled in a water bath to 37 C. Then one drop of inoculum culture that had been diluted 1:10 with 0.85% saline solution was added and the samples incubated at 37 C for 24 hours.

Measurement of growth response: All steps were performed as described for S. zymogenes with the exception of wavelength, which was set at 700 mμ for this bacterium (Difco Manual, 1969).

Calculation of results were performed as described under S. zymogenes (page 10).

III. Method using Leuconostoc mesenteroides P-60 for "total" cystine

For "total" cystine acid hydrolysis was used. Since L. mesenteroides does not have proteolytic characteristics it barely grows on enzymatic digests. These enzymatic digests are mainly composed of peptides and low molecular proteins which have to be broken down by bacterial enzymes. Only S. zymogenes has such enzymes. There is no known proteolitic bacteria for which cystine is an essential amino acid. Therefore the determination of "total" cystine in this study was restricted.

For cystine the oxidized peptone medium of Lyman et al. (1946) in combination with the basal medium made up as recommended by Evans and Bauer (1971) was used.

Reagents

Composition and preparation see Appendix 5.

Preparation of the samples for test: The hydrolysates used for "total" methionine with S. zymogenes were used for L. mesenteroides.

Determination of "total" cystine: Triplicate portions of 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, and 5 ml of standard cystine (2 µg l-cystine/ml) were distributed into 18 x 150 mm test tubes. Duplicate 0.4, 1, 1.6 ml portions of each sample were pipetted into the same sized test tubes. Five ml of the prepared cystine assay medium was added to each tube and brought to a final volume of 10 ml with distilled water. Each tube was covered with a test tube cap and sterilized by autoclaving at exactly 15 pounds pressure (121 C) for only 8 minutes. Inoculation, measurement of growth response, and calculation of results were performed as previously described under S. zymogenes (page 9).

IV. Materials and preparations of potato samples

For the evaluation of methods to determine the sulfur containing amino acids, the following potato clones were chosen: Russet Burbank, Merrimack 58, Michigan State 321-65, 322-6, 709 and 711-3. The potatoes were grown on the Montcalm Experimental Farm in 1969 on a Montcalm sandy loam, fertilized with 128 lbs/A of N, 192 lbs/A of P_2O_5 and 192 lbs/A of K_2O. Eight inches of water was added as irrigation. After harvest the potatoes were stored at 40 F until April 1970.

Preparation of samples: From each variety 8 lbs of tubers was randomly chosen, thoroughly washed, and sliced into 1 to 2 mm thick slices and placed immediately on a dry

ice layer where they froze completely within 3 to 5 minutes. The samples were kept frozen at -20 C until they could be freeze dried.

After freeze drying the samples were ground through an 80 mesh sieve in a Wiley mill and stored in screw cap bottles at a temperature lower than -5 C.

For the analysis of a segregating population comprising 834 clones from 18 families, 180 samples were randomly chosen. These potatoes were grown on the Montcalm Experimental Farm in 1970 on a Montcalm sandy loam with 800 lbs of 14-14-14 fertilizer at planting time, 130 lbs of N were added as a sidedressing and when spraying for insects and diseases. Irrigation consisted of an added 8 inches of water and after harvest the potatoes were stored at 40 F until November 1970.

Preparation of samples: From each clone 5 tubers were chosen randomly. They were thoroughly washed, cut longitudinally in two parts and from one part 1 to 2 mm thick slices were removed with a potato chip slicer. The slices were put immediately on a layer of dry ice where they froze within 3 to 5 minutes.

The samples, consisting of 35 slices of each clone, were kept in a freezer at -20 C until they could be freeze dried at a plate temperature below 100 F for 48 hours.

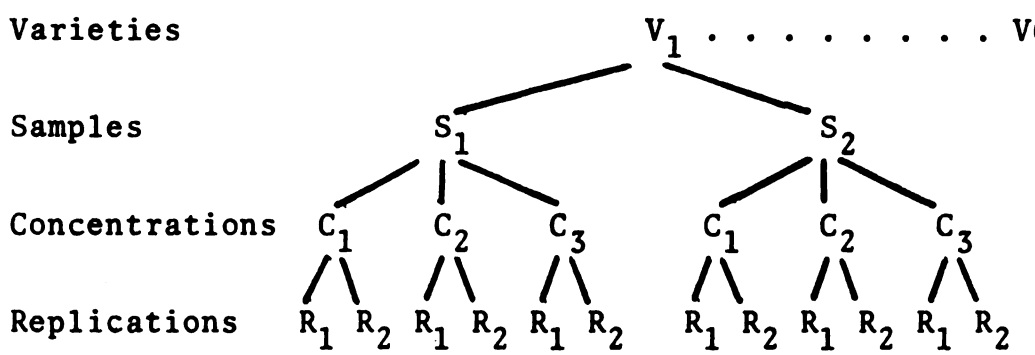
After freeze drying and grinding in a Wiley mill through an 80 mesh sieve, the samples were stored in screw cap bottles at -5 C.

RESULTS AND DISCUSSION

Criteria chosen to judge the different procedures

For uniform conditions the following statistical hierarchic assay structure was applied:

Figure 1: Hierarchic assay structure



Model: $Y_{ijkl} = \mu + \alpha_i + S_{ij} + C_{ijk} + \varepsilon_{ijkl}$

where:

Y_{ijkl} is the lth observation in the kth concentration in the jth sample of the ith variety

μ is the parametric mean of the population

α_i is the fixed variety effect

S_{ij} is the random contribution for the jth sample of ith variety

C_{ijk} is the random contribution for the kth concentration in the jth sample of the ith variety

ε_{ijkl} is the error term of the measurement of the lth test tube in the kth concentration in the jth sample of the ith variety

It is assumed that: $S_{ij} \sim N(0,\sigma_S^2)$

$C_{ijk} \sim N(0,\sigma_C^2)$

$\varepsilon_{ijk} \sim N(0,\sigma^2)$

and α_i are fixed

$i = 1 \ldots\ldots v$, $v = 6$ (varieties)

$j = 1 \ldots\ldots s$, $s = 2$ (samples per variety)

$k = 1 \ldots\ldots c$, $c = 3$ (concentrations within one sample)

$l = 1 \ldots\ldots r$, $r = 2$ (replications within one concentration level)

The basic computations were conducted as described by Sokal and Rohlf (1969 a).

During this study the time required for analyses was recorded.

Table 2. Methionine analysis of 6 cultivars conducted with L. mesenteroides P-60, acid hydrolysis

Source	df		MS	FS	EMS
Among varieties	5	2.0708	0.4142(1)	44.54***	$\sigma^2 + 2\sigma_C^2 + 6\sigma_S^2 + 12K_V^2$
Among samples	6	0.0559	0.0093(2)	1	$\sigma^2 + 2\sigma_C^2 + 6\sigma_S^2$
Among concentrations	24	0.2541	0.0106(3)	2.94*	$\sigma^2 + 2\sigma_C^2$
Within concentrations	36	0.1302	0.0030(4)		σ^2

*$\alpha = 0.05$ **$\alpha = 0.01$ ***$\alpha = 0.001$

α = Probability of error

From the components of the expected mean squares shown in Table 2 the single variance components can be derived:

$\hat{\sigma}^2 = MS\ (4)$ equation (1)

$\hat{\sigma}_C^2 = [MS\ (3) - MS\ (4)]/2$ equation (2)

$\hat{\sigma}_S^2 = [MS\ (2) - MS\ (3)]/6$ equation (3)

$K_V^2 = [MS\ (1) - MS\ (2)]/12$ equation (4)

in numbers:

equation (1): $\hat{\sigma}^2 = 0.00362$

equation (2): $\hat{\sigma}_C^2 = 0.00349$

equation (3): $\hat{\sigma}_S^2 = 0$

equation (4): $K_V^2 = 0.3347$

or expressed as percentages of a variety mean:

$\hat{\sigma}_{\bar{y}}^2 = 0.000884 = 100$ % variance of a variety mean

$\hat{\sigma}^2/scr = 0.000302 = 34.16$% variance within concentrations

$\hat{\sigma}_C^2/sc = 0.000582 = 65.84$% variance among concentrations

$\hat{\sigma}_S^2/s = 0 = 0$ % variance among samples

Significant differences among varieties

Tukey's multiple comparison procedure was chosen for this purpose:

Least significant range: $LSR_\alpha = Q_{(\nu,k)} \times s\bar{y}$

$\nu = n-1 = 11, \quad k = 6, \quad Q_{\alpha = 0.01(11,6)} = 6.247$

$s_{\bar{y}} = \sqrt{MS(2)/n} = \sqrt{0.0093/12} = 0.028$

$LSR_{\alpha = 0.01} = 6.247 \times 0.028 = 0.175$

Results: µg methionine per mg d.m. of 6 cultivars

Merrimack 58	M.S. 711-3	Russet Burbank	M.S. 709	M.S. 322-6	M.S. 321-05
1.46	1.15	1.13	1.11	1.0	0.91

*

*α = 0.01 = Probability of error

Table 3: Recovery control of methionine

Materials	Added methionine mg/g d.m.	Recovered methionine mg/g d.m.	Recovery in %
Potato starch*	1	0.8	80%
Merrimack .58 potato flow	0.5	0.48	96%

*mean of two tests

The recovery of methionine added before hydrolysis was better in potato flour (Merrimack 58) than in pure potato starch.

Optimal allocation of resources

The assay structure was reorganized to determine the most efficient combination to give a variance less than or equal to a selected variance of the variety mean.

equation (6) $\hat{\sigma}^{2'}_{\bar{y}} = \frac{d^2}{2t^2}$ $\hat{\sigma}^{2'}_{\bar{y}}$ = estimated variance of a variety mean

equation (6) $\hat{\sigma}^{2'}_{\bar{y}} = \frac{0.297^2}{2 \times 2.1^2} = 0.01$ $t = 2.1$

$d = 0.297$ = least significant difference between two varieties

Computations of total time C_0, s', c', and r'

Sokal and Rohlf (1969 b) give the equations as shown below.

$$C_0 = c_1 s' + c_2 s'c' + c_3 s'c'r' \qquad \text{equation (7)}$$

$$\hat{\sigma}^2_{\bar{y}} = \hat{\sigma}^2_s / s' + \hat{\sigma}^2_c / s'c' + \sigma^2 / s'c'r' \qquad \text{equation (8)}$$

$$s' = 1/\hat{\sigma}^2_{\bar{y}} \left[\hat{\sigma}^2_S + \hat{\sigma}^2_C / c' + \hat{\sigma}^2 / c'r'\right] \qquad \text{equation (9)}$$

$$c' = \sqrt{c_1 \hat{\sigma}^2_C / c_2 \hat{\sigma}^2_S} \qquad \text{equation (10)}$$

$$r' = \sqrt{c_2 \sigma^2 / c_3 \sigma^2_C} \qquad \text{equation (11)}$$

c_1, c_2 and c_3 are defined on page 32 and their values are given in Table 9. $\hat{\sigma}^2$, $\hat{\sigma}^2_C$, and $\hat{\sigma}^2_S$ could be calculated from the analysis of variance tables. Finally, s', c', and r' are the new numbers of samples per variety, concentrations within one sample, and replications within one concentration level, respectively.

equation (10): $c' = \sqrt{15 \times 0.0349 / 1 \times 0}$ = "big" $c' \rightarrow 2$

equation (11): $r' = \sqrt{1 \times 0.00362 / 3 \times 0.00349}$ = 0.58 $r' \rightarrow 1$

equation (9): $s' = 1/0.01[0+0.00349/2+0.00362/2] = 0.35 \quad s' \to 1$

equation (7): $C_0 = 15 \times 1 + 1 \times 2 + 4 \times 2 = 25$ min.

In order to check the variance we take equation (8):

$$\hat{\sigma}^{2'}_{\bar{y}} = 0/1 + 0.00349/2 + 0.00362/2 = 0.00174$$

$\hat{\sigma}^{2'}_{\bar{y}} = 0.00174$, smaller than requested (0.01)

The new assay structure would comprise:

1 sample (s')

2 concentration levels per sample (c')

1 replication per concentration level (r')

The time involved for analysis of one variety after optimal allocation would be 25 minutes, compared to 84 minutes using the basic structure as given on page 16.

Methionine analysis of 6 cultivars conducted with S. zymogenes, acid hydrolysis

Assay structure, model and assumptions of the analysis of variance are the same as given on page 16.

Table 4: Analysis of variance of methionine content estimated by means of S. zymogenes, acid hydrolysis

Source	df	SS	MS	FS	EMS
Among varieties	5	2.4130	.4826(1)	41.25***	$\sigma^2 + 2\sigma^2_C + 6\sigma^2_S + 12K^2_V$
Among samples	6	.0699	.0117(2)	1.26	$\sigma^2 + 2\sigma^2_C + 6\sigma^2_S$
Among concentrations	24	.223	.0093(3)	3.1**	$\sigma^2 + 2\sigma^2_C$
Within concentrations	36	.1086	.003 (4)		σ^2

From the components of the expected mean squares shown in Table 4 the single variance components can be derived analogically as in test 1:

equation (1): $\hat{\sigma}^2 = 0.003$

equation (2): $\hat{\sigma}_C^2 = 0.0032$

equation (3): $\hat{\sigma}_S^2 = 0.004$

equation (4): $K_V^2 = 0.0392$

Expressed as percentages of a variety mean:

equation (5): $\hat{\sigma}_{\bar{y}}^2 = 0.00098 = 100$ % variance of a variety mean

$\hat{\sigma}^2/scr = 0.00025 = 25.5$ % variance within concentrations

$\hat{\sigma}_C^2/sc = 0.00053 = 54.08$% variance among concentrations

$\hat{\sigma}_S^2/s = 0.0002 = 20.41$% variance among samples

Significant differences among varieties

Here again Tukey's multiple comparison procedure has been chosen.

$LSR_\alpha = Q_{\alpha(\nu,k)} \times s_{\bar{y}}$

$Q_\alpha = 0.01(11,6) = 6.247$

$s_{\bar{y}} = \sqrt{MS(2)/n} = \sqrt{0.0117/12} = 0.031$

$LSR_{\alpha = 0.01} = 6.247 \times 0.031 = 0.194$

Results: µg methionine per mg d.m. 6 cultivars

Merrimack 58	M.S. 711-3	M.S. 709	Russet Burbank	M.S. 322-6	M.S. 321-65
1.74	1.73	1.47	1.42	1.35	1.25

*

*α = .01

Table 5: Recovery control of methionine

Materials	Added methionine mg/g d.m.	Recovered methionine mg/g d.m.	Recovery in %
Potato starch	2	2.02	101
M.S. 321-65 flour	1	1.06	106

Table 5 shows a satisfactory recovery of methionine.

Optimal allocation of resources

The analogue calculations as on pages 20 and 21 were performed with the values obtained from this method:

$\hat{\sigma}^{2'}_{\bar{y}}$ remains 0.01

equation (10): $c' = 3.5$, $c' \rightarrow 2$

equation (11): $r' = 0.484$, $r' \rightarrow 1$

equation (9) : $s' = 0.71$, $2' \rightarrow 1$

equation (7) : C_0 = 25 minutes

In order to recheck the variance take:

equation (8) : $\hat{\sigma}^{2'}_{\bar{y}} = 0.0071$, smaller than required (0.01)

The new assay structure, given a maximal variance of a mean ($\hat{\sigma}^{2'}_{\bar{y}} \leq 0.01$), would comprise

1 sample (s')

2 concentration levels per sample (c')

1 replication per concentration level (r')

and the time involved for the analyses of one variety after optimal allocation of resources would be 25 minutes compared to 84 minutes using the basic structure as given on page 16.

Methionine analysis of 6 cultivars using S. zymogenes, and enzymatic digestion

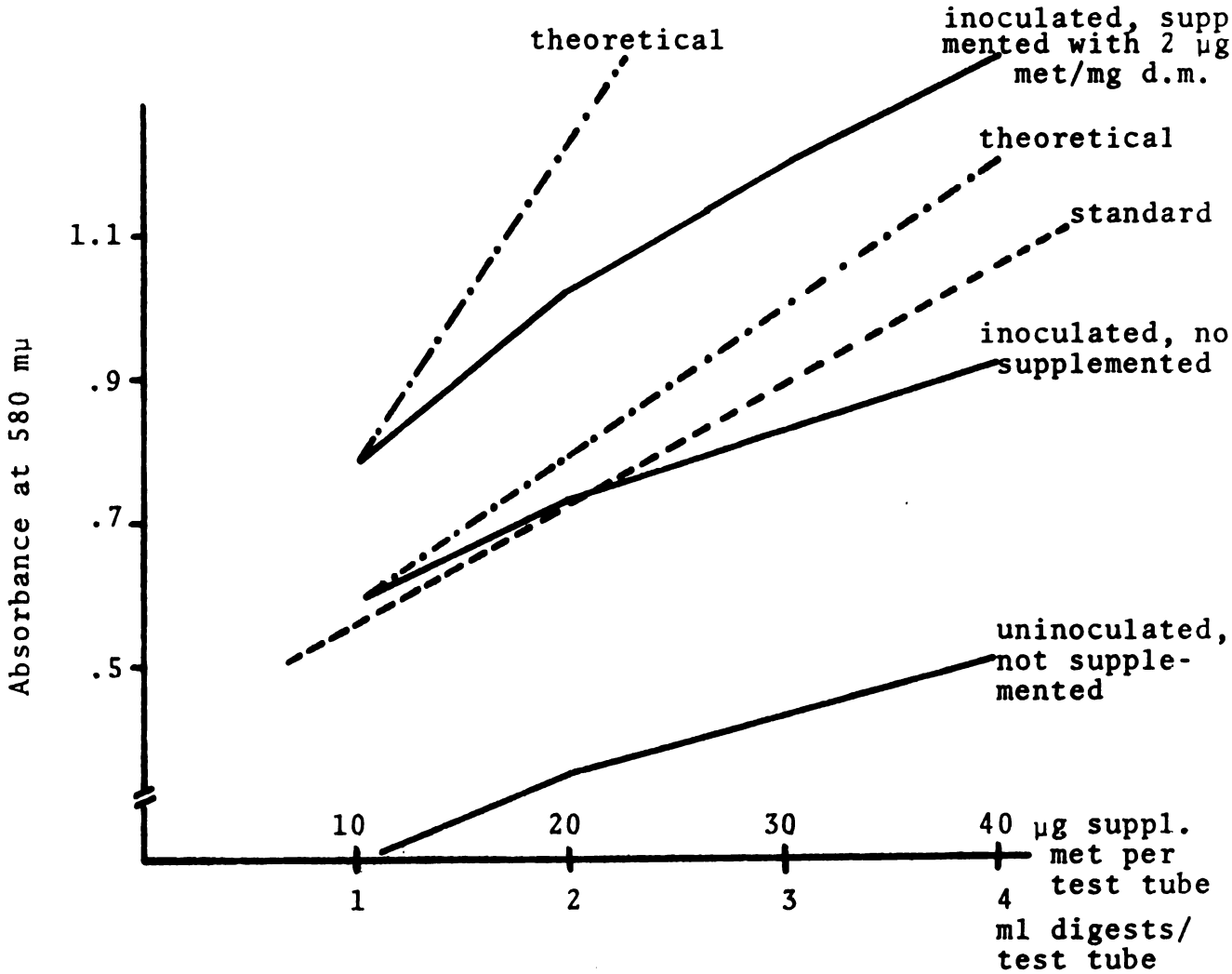
In contrast to the acid hydrolysates, the enzymatic digests were not filtered and were therefore turbid, even when diluted in the test tubes.

An attempt was made to correct this basic turbidity. Some tests have shown that the basic turbidity of digested potato starch samples and digested potato samples is basically the same.

As concentrations of potato starch digests increased, the relative absorbance became smaller. This was true for both, the inoculated and the uninoculated starch samples (Figure 2).

To correct both, the basic turbidity and the decreased absorbance at higher concentration levels, the inoculated starch sample at a given concentration was subtracted from the same concentration of a sample with methionine

Figure 2: Absorbance curve of uninoculated and inoculated potato starch samples partially supplemented with methionine



supplemented potato starch (Table 6). This procedure also included the correction for methionine contained in the 4% papain enzyme solution.

Table 6: Procedure to correct basic turbidity

Digests per test tube		Inoculated potato starch supplemented with 2 µg met/mg d.m.		Inoculated potato starch		
ml	mg d.m.	Absorbance 580 mµ	A = gross met, µg/mg d.m.	Absorbance 580 mµ	B = tare met, µg/mg d.m.	A-B = net met µg/mg d.m.
1	5	.77	3.95	.6	2.05	1.90
1	5	.79		.59		
2	10	1.03	3.68	.75	1.78	1.90
2	10	1.025		.73		
3	15	1.205	3.42	.84	1.5	1.92
3	15	1.2		.835		
4	20	1.33	3.22	.93	1.47	1.75
4	20	1.36		.9		

Recovery of methionine from the supplemented starch samples was 93.5%.

In all tests where the "available" methionine was determined, two potato starch samples were prepared to correct the gross value of the potato samples.

Table 7: Analysis of variance of methionine content estimated with S. zymogenes, enzymatic digestion

Source	df	SS	MS	F_S	EMS
Among varieties	5	3.381	0.6762(1)	15.333**	$\sigma^2 + 2\sigma_C^2 + 6\sigma_S^2 + 12K_V^2$
Among samples	6	0.2644	0.0441(2)	1	$\sigma^2 + 2\sigma_C^2 + 6\sigma_S^2$
Among concentrations	24	1.4056	0.0586(3)	9.849***	$\sigma^2 + 2\sigma_C^2$
Within concentrations	36	0.2142	0.00595(4)		σ^2

From the components of the expected mean squares shown in Table 7 the single variance components can be derived:

equation (1) = $\hat{\sigma}^2 = 0.00595$

equation (2): $\hat{\sigma}_C^2 = 0.0263$

equation (3): $\hat{\sigma}_S^2 = 0$

equation (4): $K_V^2 = 0.0392$

Expressed as percentages of a variety mean:

equation (5): $\hat{\sigma}_{\bar{y}}^2 = 0.004819 = 100$ % variance of a variety mean

$\hat{\sigma}^2/scr = 0.000496 = 10.51$% variance within concentrations

$\hat{\sigma}_C^2/sc = 0.004383 = 89.83$% variance among concentrations

$\hat{\sigma}_S^2/s = 0 = 0$ % variance among samples

Significant differences

Tukey's multiple comparison procedure has been applied:

$$LSR_{\alpha} = Q_{\alpha(\nu,k)} \times s\bar{y}$$

$$Q_{\alpha} = 0.01\ (11,6) = 6.247$$

$$s\bar{y} = \sqrt{MS(2)/n} = \sqrt{0.00441/12} = 0.0606$$

$$LSR_{\alpha = 0.01} = 6.247 \times 0.0606 = 0.379$$

Results: µg methionine per mg d.m. of 6 cultivars

Merrimack 58	M.S. 711-3	M.S. 322-6	Russet Burbank	M.S. 321-65	M.S. 709
* 1.86	1.53	1.53	1.36	1.33	1.17

*α = .01

Table 8: Recovery control of methionine

Materials	Added methionine mg/g d.m.	Recovered methionine mg/g d.m.	Recovery in %
Potato starch	2	2.09	104
Merrimack 58 flour	1	.93	93

Table 8 shows that the recovery of methionine was within reasonable limits.

Optimal allocation of resources

The analogue calculations as on pages 20 and 21 were performed with the values obtained from this method.

With given value for $\hat{\sigma}^{2'}_{\bar{y}} = 0.01$

equation (10): c' = "big" , c' → 3

equation (11): r' = 0.24 , r' → 1

equation (9): s' = 1.075 , s' → 1

equation (7): C_0 = 20 minutes

In order to recheck the variance we take equation (8):

$$\hat{\sigma}^{2'}_{\bar{y}} \quad 0.0175 \approx 0.01$$

The new assay structure would comprise:

1 sample (s')

3 concentration levels per sample (c')

1 replication per concentration level (r')

and the time involved for the analysis of one variety after optimal allocation of resources would be 20 minutes compared to 64 minutes following the basic structure as given on page 20 and 21.

Figure 3: Cultivar means of methionine of all methods

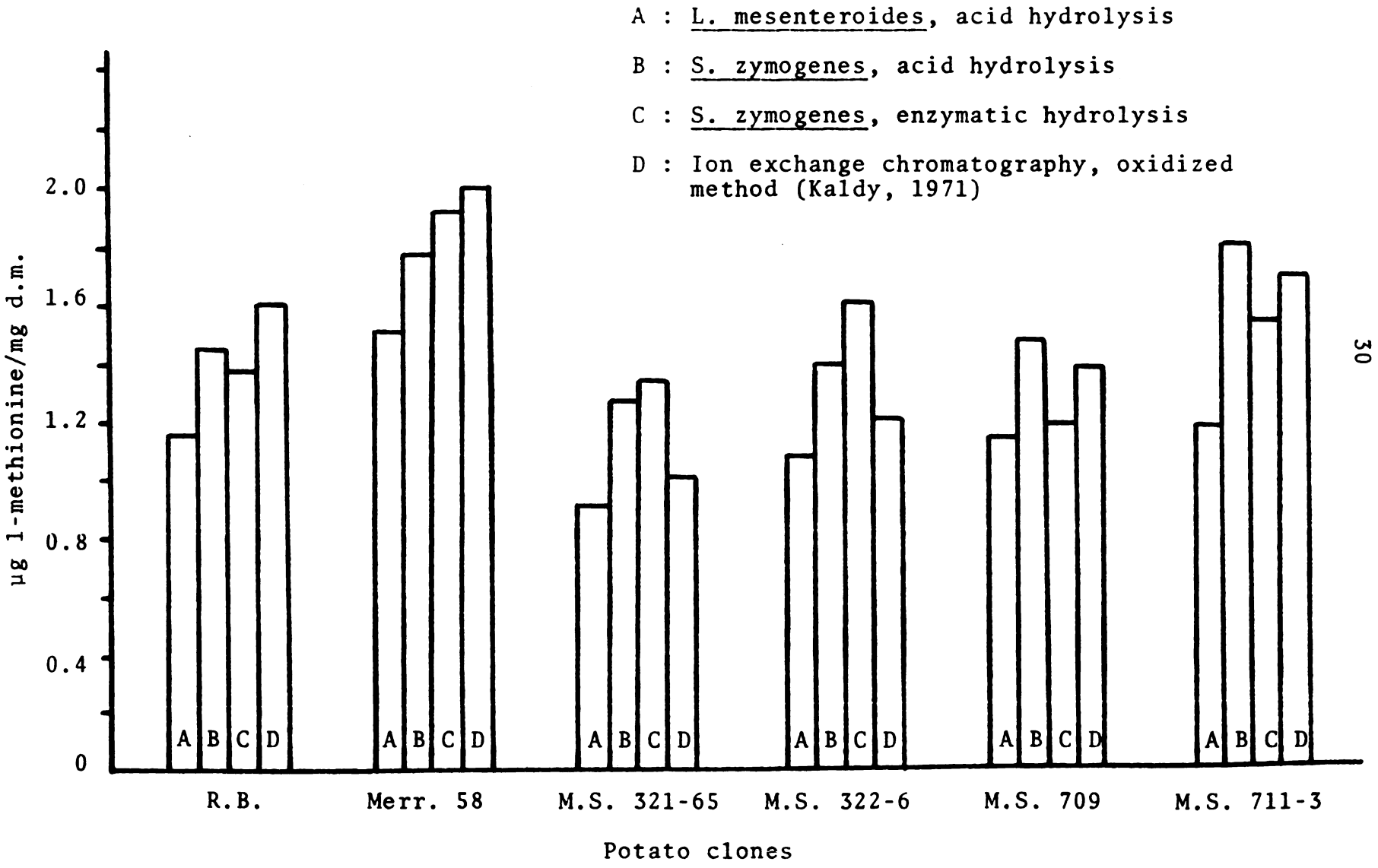


Figure 4: Cultivar means of cystine

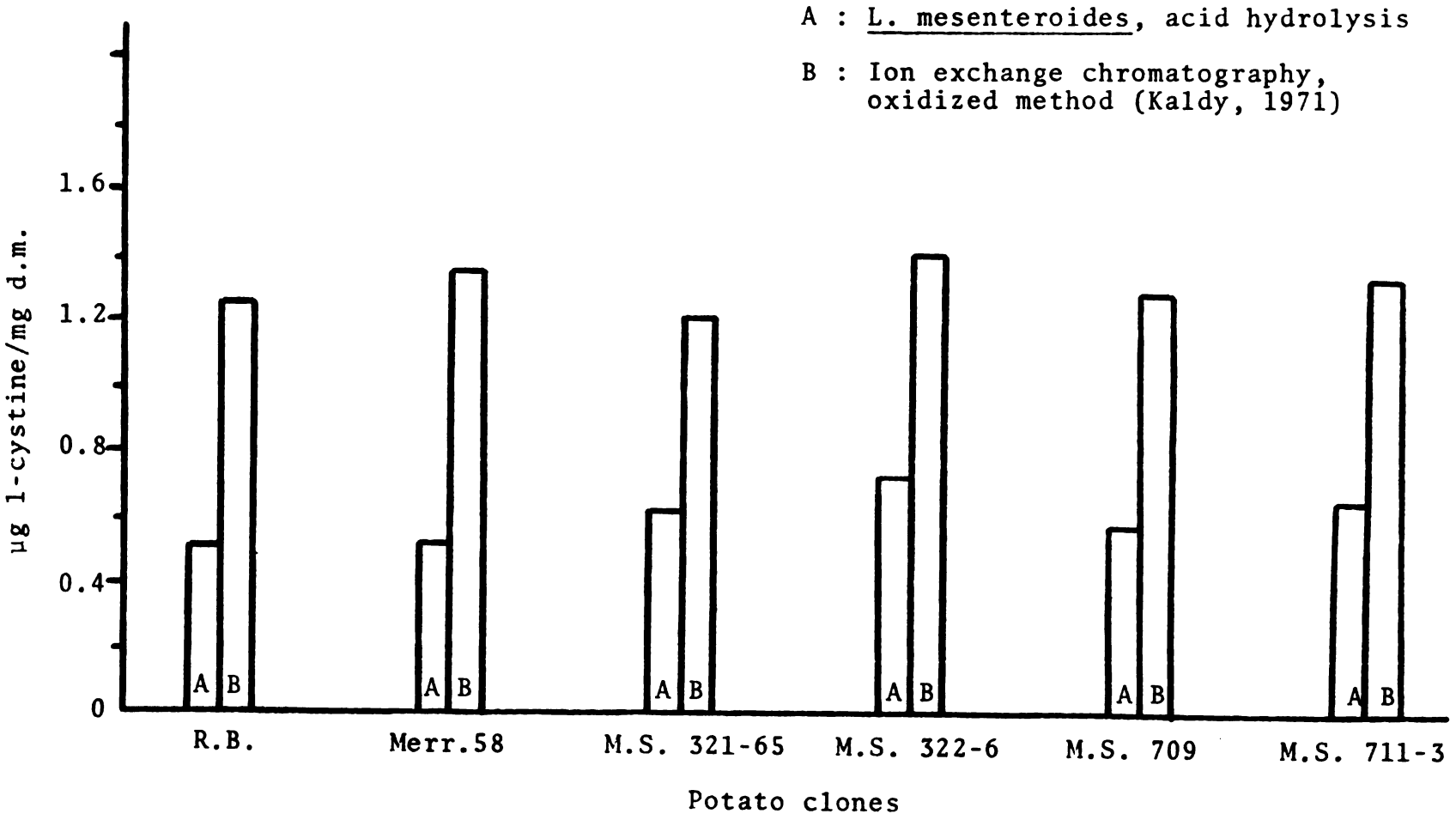


Table 9: Summarized results, part I

Criteria	Time required per level unit in minutes			Variance components of a variety mean					
				absolute			in %		
	T.1	T.2	T.3	T.1	T.2	T.3	T.1	T.2	T.3
Variety				0.000884	0.00098	0.00482	100	100	100
Samples	15*	15*	5*	0	0.0002	0	0	20.41	0
Concentrations	1**	1**	1**	0.000582	0.00053	0.00438	65.84	54.08	89.83
Replications	4***	4***	4***	0.000302	0.00025	0.00050	34.16	25.51	10.17

*c_1: (includes time needed to weigh and prepare the hydrolysate of 1 sample)

**c_2: (includes additional time required for 1 concentration level)

***c_3: (includes time needed to pipette, sterilze, read the absorbance, and calculate the methionine content of 1 replication)

T.1 : methionine analysis using L. mesenteroides and acid hydrolysis

T.2 : methionine analysis using S. zymogenes and acid hydrolysis

T.3 : methionine analysis using S. zymogenes and enzymatic hydrolysis

Table 10: Summarized results, part II

Criteria		T.1	T.2	T.3
Variance of a variety mean		0.028	0.031	0.0606
Recovery in %	in potato starch	80	101	104
	in whole potato	96	106	93
Total time needed for 1 variety in min.	original structure	84	84	64
	optimally allocated resources*	25	25	20
	optimally allocated resources**	25	25	15

*condition : variance of a variety mean $\hat{\sigma}^2_{\bar{y}} \leq 0.01$

equation (6) least significant difference $d \leq \sqrt{0.088} = 0.297$

**condition : variance of a variety mean $\hat{\sigma}^2_{\bar{y}} \leq 0.02$

equation (6) least significant difference $d \leq \sqrt{0.176} = 0.42$

Discussion and conclusions of methods evaluating methionine

Generally speaking all methionine determinations by S. zymogenes were higher than those by L. mesenteroides P-60 (Figure 3). Better recovery of supplemented methionine, which ranged from -3 to 24%, could be a partial explanation (Table 10). The overall means in T.1, T.2, and T.3 were 1.13, 1.49 and 1.47 µg methionine per mg d.m., respectively. A 24% increase of the mean value of T.1 would yield 1.40 µg methionine which is still beyond those of T.2 and T.3.

On the other hand, differences have been reported due to different bacteria species.

However in T.1 and T.2 the results of the single varieties are proportional.

The results obtained with enzymatic digestion were lower but not exactly proportional as would be expected when the difference was considered in digestibility or biological availability of the individual amino acids from clone to clone.

It was surprising to observe that the assay mean of T.3 (1.47 µg met/mg d.m.) was almost as high as in T.2 (1.49 µg met/mg d.m.) since the "available" methionine was expected to be lower than the "total" methionine. But what is the actual methionine content? This question arose when the methionine evaluated by microbiological procedures was compared to that evaluated by chemical methods (Kaldy, 1971). Here, with the exception of cultivars 322-6 and 321-65, all values were higher than those evaluated in T.3. This was to be expected since methionine was oxidized before the acid hydrolysis and all methionine should have been recovered.

In four cases the results estimated by chromatography and those by S. zymogenes and acid hydrolysis were within a range of 10%. In all cases L. mesenteroides gave the lowest results.

The potato tuber contains some methionine sulfoxide in its tissue (Dent et al., 1947; Payne et al., 1951).

Furthermore it has been reported (Block, 1956 a) that during acid hydrolysis some methionine would be oxidized to methionine sulfoxide. This oxidized methionine probably was included in the results evaluated by the ion chromatographic procedure of Kaldy (1971). Possibly, some bacteria can utilize methionine sulfoxide. The recovery from S. zymogenes for both, the supplemented potato starch and whole potato (Table 10) was better than from L. mesenteroides. Therefore it was possible that S. zymogenes utilized methionine sulfoxide while L. mesenteroides did not.

This hypothesis would explain the fact that the results were quite close when estimated by S. zymogenes (acid hydrolysis) and ion exchange chromatography (Kaldy, 1971).

From the point of view of economics S. zymogenes (enzymatic digestion) needs only 60% of the time required for L. mesenteroides and is more efficient (at a given $\hat{\sigma}^2_{\bar{y}} \leq 0.02$). This is clearly shown in Table 10.

Cystine analysis conducted by using L. mesenteroides P-60, acid hydrolysis

Assay structure, model and assumptions for analysis of variance are the same as given on page 16.

Table 11: Analysis of variance of cystine estimated by using L. mesenteroides P-60, acid hydrolysis

Source	df	SS	MS	F_S	EMS
Among varieties	5	0.4526	0.0904 (1)	97.2***	$\sigma^2 + 2\sigma_C^2 + 6\sigma_S^2 + 12K_V^2$
Among samples	6	0.0056	0.00093(2)	1	$\sigma^2 + 2\sigma_C^2 + 6\sigma_S^2$
Among concentrations	24	0.0999	0.0042 (3)	1.5	$\sigma^2 + 2\sigma_C^2$
Within concentrations	36	0.1021	0.0028 (4)		σ^2

From components of the expected mean squares shown in Table 11, one can derive the single variance components as in test 1.

equation (1): $\hat{\sigma}^2 = 0.0028$

equation (2): $\hat{\sigma}_C^2 = 0.0007$

equation (3): $\hat{\sigma}_S^2 = 0$

equation (4): $K_V^2 = 0.0179$

Expressed as percentages of a variety mean:

equation (5):

$\hat{\sigma}_{\bar{y}}^2 = 0.00035 = 100$ % variance of a variety mean

$\hat{\sigma}^2/scr = 0.00023 = 65.71\%$ variance within concentrations

$\hat{\sigma}_C^2/sc = 0.0007 = 34.29\%$ variance among concentrations

$\hat{\sigma}_S^2/s = 0 = 0$ % variance among samples

Significant differences among varieties

The multiple comparison procedure of Tukey has been chosen for this purpose.

Least significant range, $LSR_{\alpha} = Q_{\alpha(\nu,k)} \times s\bar{y}$

$k = 6$

$\nu = 11$ $\qquad Q_{\alpha} = 0.01(11,6) = 6.247$

$s\bar{y} = \sqrt{MS(2)/n} = \sqrt{0.00093/12} = 0.0088$

$LSR_{\alpha = 0.01} = 6.247 \times 0.0088 = 0.05497$

Results: µg cystine per mg d.m. of 6 cultivars

M.S. 322-6	M.S. 711-3	M.S. 321-65	M.S. 709	Russet Burbank	Merrimack 58
0.72	0.65	0.61	0.56	0.5	0.5

*

*$\alpha = 0.01$

Table 12: Recovery control of cystine

Materials	Added cystine mg/g d.m.	Recovered cystine mg/g d.m.	Recovery in %
Potato starch	1	0.54	54
Merrimack 58	0.5	0.47	94

Discussion of method evaluating cystine

It was very surprising to get such a large difference in recovered cystine between with cystine supplemented potato starch and with cystine supplemented

potato flour (Table 12). One reason for this phenomenon could be that during hydrolysis cystine was destroyed to a larger extent when it was together with pure potato starch than with all constituents of potato flour.

Two-fold differences were observed between cystine contents evaluated by using L. mesenteroides and ion exchange chromatography (Kaldy, 1970).

Values reported in the literature range from 0.8 µg cystine/100 g protein (Schuhpan and Postel, 1957) to 1.6 µg cystine per 100 g protein (Hughes, 1958). Values obtained by Kaldy (1971) were in this indicated range. However cystine evaluated in this work were slightly lower than those reported by Schuhpan and Postel (1957) who in contrast with Hughes (1958) and Kaldy (1971) did not oxidize the samples before hydrolysis.

Cystine occurs naturally in the potato tuber (Barker and Mapson, 1952; Payne et al., 1951). Since systine was oxidized to cysteine and finally to cysteic acid and determined in this latter form, the naturally occurring cysteine in its oxidized state could also be found and therefore determined as cysteic acid.

It has been reported (White et al., 1958) that bacteria used cysteine for the formation of methionine via homocystine. But since there was 20 times the necessary amount of methionine for normal growth in the cystine assay media, this reaction should not occur and therefore cysteine should have been used to form methionine.

On the other hand if methionine synthesis occurred from cysteine in the bacteria it would explain the higher recovery of supplemented methionine in the whole potato since cysteine was present in the whole potato but not in pure potato starch. However, this did not explain the large differences between the two methods of evaluating cystine.

Optimal allocation of resources

For cystine it would be sufficient to detect a difference of about d = 0.14 µg cystine per mg d.m. between two varieties.

equation (6) $\hat{\sigma}^{2'}_{\bar{y}} = 0.14^2/2 \times 4.4 = 0.00227$

$d = 0.14, \quad t = 2.1$

The analysis structure which should yield a variance of a variety mean of $\hat{\sigma}^{2'}_{\bar{y}} \leq 0.00227$ would comprise:

equation (10): $c' = \sqrt{15 \times 0.007/1 \times 0}$ = "big" , $c' \to 2$

equation (11): $r' = 1$, $r' \to 1$

equation (9) : $s' = 0.788$, $s' \to 1$

equation (7) : $C_0 = 25$ minutes

In order to recheck the variance we use

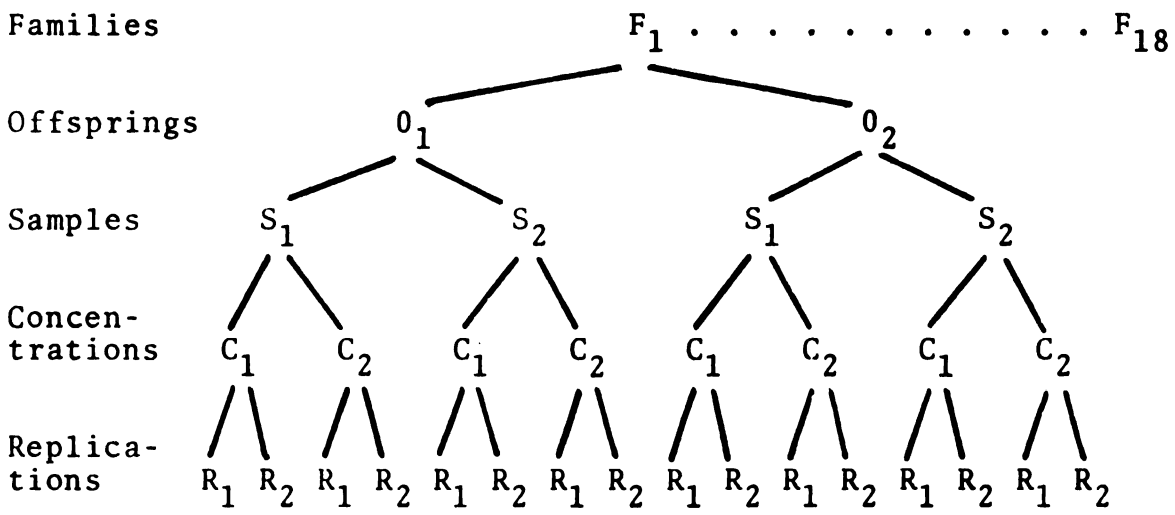
equation (8) : $\hat{\sigma}^{2'}_{\bar{y}} = 0.00175 \leq 0.00227$

$0.00175 < 0.00227$ (requested variance $\hat{\sigma}^{2'}_{\bar{y}}$)

Methionine analysis of 180 potato clones conducted by using S. zymogenes enzymatic digestion

Eighteen families with 10 clones each were tested for methionine.

Assay structure:



Model:

$$Y_{ijklm} = \mu + \alpha_i + O_{ij} + S_{ijk} + C_{ijkl} + \varepsilon_{ijklm}$$

Y_{ijklm} is the mth observation in the lth concentration of the kth sample of the jth offspring in the ith family

μ is the parametric mean of the population

α_i is the ith family effect

O_{ij} is the random contribution for the jth offspring of the ith family

S_{ijk} is the random contribution for the kth sample in the jth offspring of the ith family

C_{ijkl} is the random contribution for the lth concentration in the kth sample of the jth offspring in the ith family

ε_{ijklm} is the error term of the mth test tube's measurement of the lth concentration in the kth sample of the jth offspring in the ith family

It is assumed that:

$$\alpha_i \quad \text{are fixed}$$

$$O_{ij} \sim N(0,\sigma^2)$$

$$S_{ijk} \sim N(0,\sigma_K^2)$$

$$C_{ijkl} \sim N(0,\sigma_C^2)$$

$$\varepsilon_{ijklm} \sim N(0,\sigma^2)$$

In this test:

$i = 1 \ldots . f$, $f = 18$ (families)

$j = 1 \ldots . o$, $o = 10$ (offsprings)

$k = 1 \ldots . s$, $s = 2$ (samples)

$l = 1 \ldots . c$, $c = 2$ (concentrations)

$m = 1 \ldots . r$, $r = 2$ (replications)

This 5-level nested analysis of variance was computed from the analogue expansion of the 4-level nested analysis of variance described in Sokal and Rohlf (1969 a).

All analyses were performed in five assays. Values received were corrected by means of a standard variety analyzed in each assay. The media used was prepared at one time and kept frozen until used. Samples whose concentration values differed more than 15% were repeated. The same is true for differences between the two samples of one variety.

Table 13: Analysis of variance of methionine contents of 18 families with 10 offsprings each by using S. zymogenes, enzymatic digestion

Source	df	SS	MS	F_S	EMS
Among families	17	74.2451	4.3673(1)	2.2564**	$\sigma^2 + 2\sigma_C^2 + 4\sigma_S^2 + 8\sigma_O^2 + 80\ K_F^2$
Among offsprings	162	313.5624	1.9355(2)	22.9324***	$\sigma^2 + 2\sigma_C^2 + 4\sigma_S^2 + 8\sigma_O^2$
Among samples	180	15.1991	0.0844(3)	1.0368	$\sigma^2 + 2\sigma_C^2 + 4\sigma_S^2$
Among concentrations	360	29.3282	0.0814(4)	10.4358***	$\sigma^2 + 2\sigma_C^2$
Within concentrations	720	5.6352	0.0078(5)		σ^2

The results were calculated as described in test 3.

From the components of the expected mean squares shown in Table 13 the single variance components can be derived:

$$\hat{\sigma}^2 = MS\ (5) = 0.0078$$

$$\hat{\sigma}_C^2 = [MS\ (4) - MS\ (5)]/2 = 0.0368$$

$$\hat{\sigma}_S^2 = [MS\ (3) - MS\ (4)]/4 = 0.0008$$

$$\hat{\sigma}_O^2 = [MS\ (2) - MS\ (3)]/8 = 0.2319$$

$$K_F^2 = [MS\ (1) - MS\ (2)]/80 = 0.0300$$

or expressed as percentages of the whole assay mean:

$$\hat{\sigma}_{\bar{y}}^2 = \hat{\sigma}^2/foscr + \hat{\sigma}_C^2/fosc + \hat{\sigma}_S^2/fos + \hat{\sigma}_O^2/fo + K_F/f$$

from this equation:

$\hat{\sigma}_{\bar{y}}^2$ = 0.003017 = 100 % variance of the whole assay mean

$\hat{\sigma}^2/foscr$ = 0.000005 = 0.17% variance within concentrations

$\hat{\sigma}_C^2/fosc$ = 0.000050 = 1.66% variance among concentrations

$\hat{\sigma}_S^2/fos$ = 0.000002 = 0.07% variance among samples

$\hat{\sigma}_O^2/fo$ = 0.001290 = 47.76% variance among offsprings

K_F^2/f = 0.00167 = 55.35% variance among families

Discussion of methionine analysis of 180 potato clones

a. Analysis procedures

Table 10 clearly shows the importance of concentrations in the analysis. The extra samples contributed but 0.07% of the whole mean variance while variance among concentrations was more than 20 times greater. Therefore the proposed analysis structure for S. zymogenes and enzymatic digestion on page 29 is satisfactory provided priority is given to the number of concentrations rather than the number of samples.

b. Potato genetics

These results indicate two very clear conclusions:

1. The methionine content of families varies widely.
2. Differences in methionine within families are great (Figure 6).

Therefore it should be possible to increase methionine content in potatoes by breeding.

The second statement shows the direction for analysis of large numbers of progenies since there is wide segregation within a family (Figure 6).

As one can see in Figure 5 methionine varies from 1 up to 3.6 µg l-met per mg d.m. In Figure 6, when this overall frequency distribution is broken down to frequency distributions on the family level, one can see the diversity

Figure 5: Frequency distribution of methionine of 180 clones

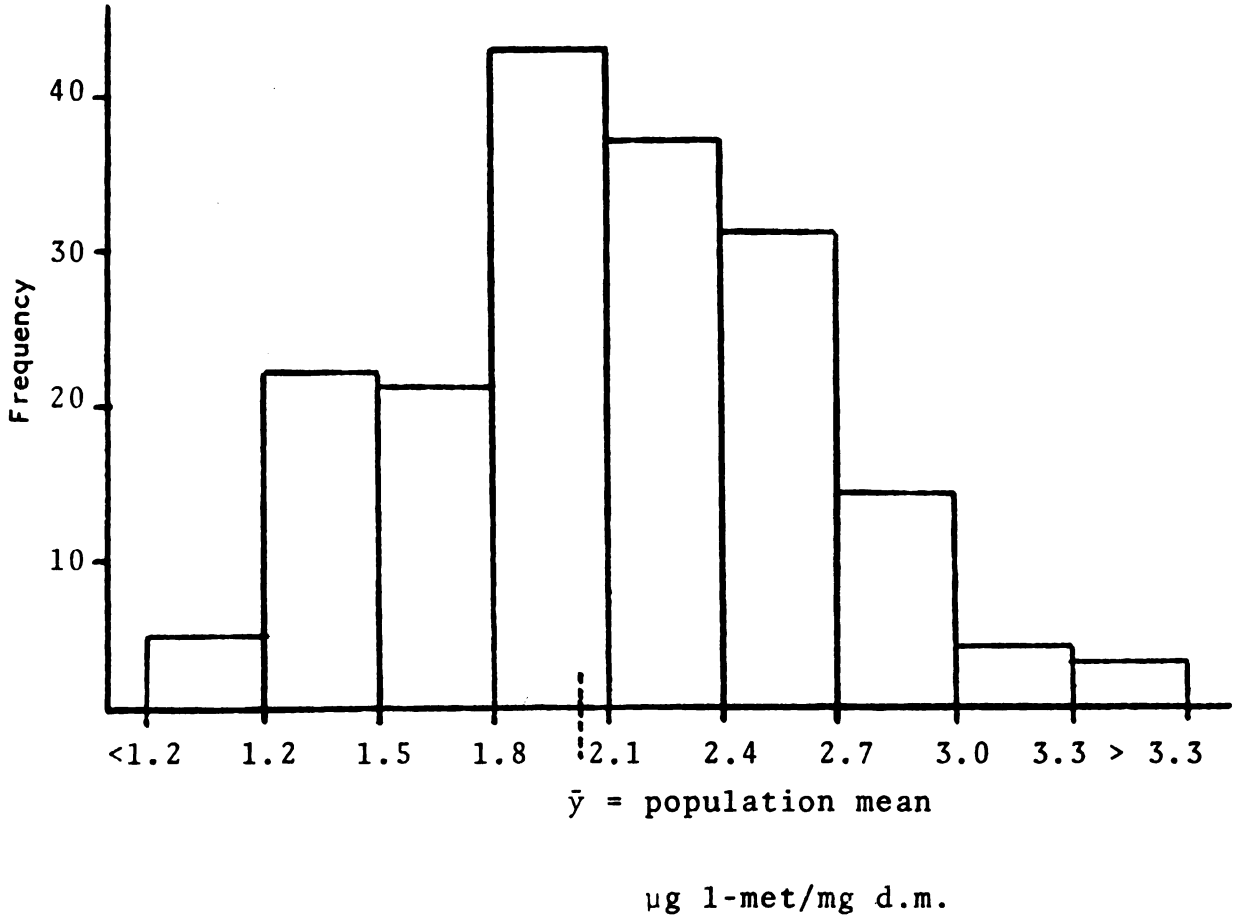


Figure 6: Frequency distributions of methionine of 180 clones according to families

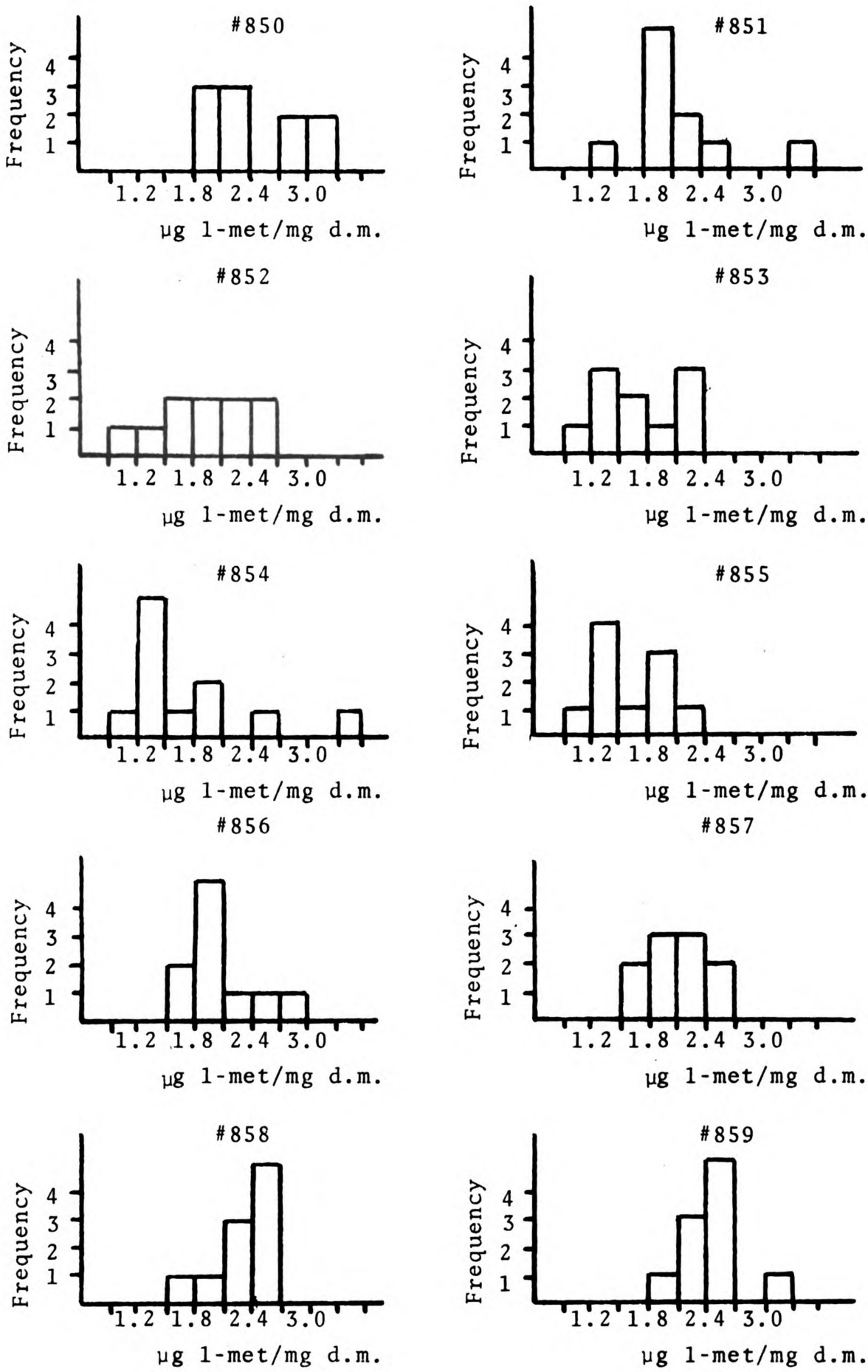
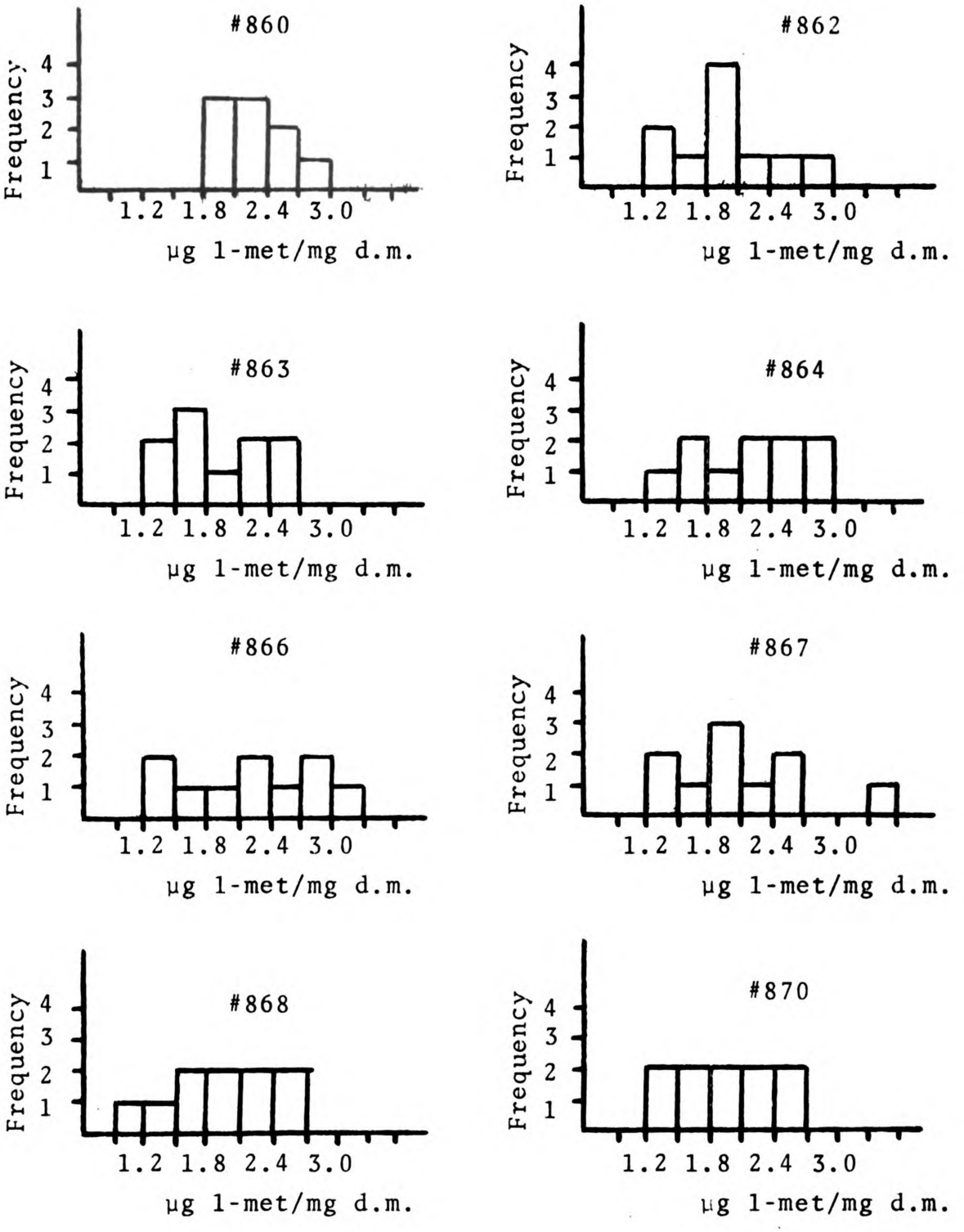


Figure 6: Cont.



of each family. For example in #857 there is little segregation which is contrary to #851, #854, #864, #866 and #867 which have clones with methionine contents ranging from 1.2 up to 3.6 µg l-met per mg d.m.

REFERENCES

Albanese, A. A., and Orto, L. A. 1968. Proteins and amino acids. In "Modern Nutrition in Health and Disease," ed. M. G. Wohl and R. S. Goodhart, p. 107. Lea and Febiger.

Barker, J., and Mapson, L. W. 1952. The absorbic acid content of potato tubers. III The influence of storage in nitrogen, air, and pure oxygen. New Phytologist 51, 90-115.

Borgstrom, G. 1969. Too Many: A Study of Earth's Biological Limitations, pp. 40-45. Macmillan Ltd., London.

Barton-Wright, E. C. 1952. The Microbiological Assay of the Vit B-Complex and Amino Acids. Pitman, London.

Block, R. J. 1956 a. Amino Acid Handbook, pp. 131, 132. Charles C. Thomas, Publisher, Springfield, Illinois, USA.

_____. 1956 b. Amino Acid Handbook, p. 51. Charles C. Thomas, Publisher, Springfield, Illinois, USA.

Boyne, A. W., Price, S. A., Rosen, C. D., and Stott, J. A. 1967. Protein quality of feeding-stuffs, 4., Progress report on collaborative studies on the microbiological assay of available amino acids. Br. J. Nutr., 21, 181-206.

Dent, C., Stepka, W., and Steward, F. 1947. Detection of the free amino acids of plant cells by partition chromatography. Nature, 160, 682-683.

Difco Manual of Dehydrated Culture Media and Reagents for Microbiological and Clinical Laboratory Procedures. Ninth Edition, 1969, p. 232. Difco Laboratories, Inc., Detroit, Mich.

Evans, R., and Butts, H. A. 1948. Studies on the heat inactivation of lysine in soy bean oil meal. J. biol. Chem. 175, 15-20.

Evans, R., Bandemer, S. L., and Bauer, D. H. 1962. Effect of heating soybean proteins in the autoclave on the liberation of cystine and methionine by several digestion procedures. Agricultural and Food Chemistry, 10, 416.

Evans, R. J., and Bauer, D. H. 1970. Working paper. Michigan State University, Department of Biochemistry, E. Lansing, Mich.

Food and Agriculture Organisation of the United Nations. 1957. Protein requirements. FAO Nutritional Studies No. 16, Rome.

Ford, J. E. 1960. A microbiological method for assessing the nutritional value of proteins. Br. J. Nutr. 14, 485-497.

_____. 1962. A microbiological method for assessing the nutritional value of proteins, 2., The measurement of "available" methionine, leucine, isoleucine, arginine, histidine, tryptophan, and valine. Br. J. Nutr. 16, 409-25.

Guggenheim, K. 1970. Evaluation of Novel Protein Products. Proceedings of the International Biological Program (IBP) and Wenner-Gren Center Symposium held in Stockholm, Sept. 1968, p. 238. Edited by A. E. Bender, B. Loefqvist, R. Kihlberg and L. Munck. Pergamon Press, Oxford, N.Y., Toronto, Sydney, Braunschweig.

Hughes, B. P. 1958. The amino acid composition of potato protein and of cooked potato. British J. Nutr. 12, 188-195.

Kaldy, M. S. 1971. Evaluation of the potato protein by amino acid analysis and dye-binding. Ph.D. Thesis, Michigan State University, E. Lansing, Mich.

Kidder, G. W., and Dewey, V. C. 1949. Studies on the biochemistry of Tetrahymena. XIII. B vitamin requirements. Arch. Biochem. 21, 66-73.

Lewis, O. A. M. 1966. Short ion-exchange column method for the estimation of cystine and methionine. Nature, 209, 5039-5040.

Lyman, C. M., Moseley, O., Wood, S., and Hale, F. 1946. Note on the use of hydrogen peroxide treated peptone in media for the microbiological determination of amino acids. Arch. Biochem. 10, 427-42.

Mulder, E. S., and Bakema, K. 1956. Effect of the nitrogen, phosphorus, potassium and magnesium nutrition of potato plants on the content of free amino acids and on the amino acid composition of the protein of the tubers. Plant and Soil, VII, No. 2, 135-166.

Payne, M. G., Fults, J. L., and Hay, R. J. 1951. Free amino-acids in potato tubers altered by 2, 4-D treatment of plants. Science (Washington) 114, 204-205.

Rios, B. J. 1969. Protein value of potato and navy bean powders: Nutritional evaluation using the meadow vole (Microtus pennsylvanicus), p. 49. Master Thesis, Michigan State University, E. Lansing, Mich.

Rosen, G. D., Stott, J. A., and Smith, H. 1960. Proc. int. Congr. Nutr. V. Washington, p. 72.

Schram, E., Moore, S., and Bigwood, B. J. 1954. Chromatography determination of cystine and cysteic acid. Biochem. J. 57, 33-37.

Schuhpan, W., and Postel, W. 1957. Ueber die biologische Wertigkeit des Kartoffeleiweisses. Die statistische Streuung der Anteile der exogenen Aminosaeuren am Rohprotein in Abhaengigkeit von genetischen und oekologischen Faktoren Naturwiss. 44, 40-41.

Schuhpan, W. 1958. Proteins et amino-acids. Teneurs en amino-acids indispensables des vegetaux alimentaires et de leur diverses organes. Qual. Plant. Mater. Veg. 3-4, 19-33.

Sokal, R. R., and Rohlf, F. J. 1969 a. Biometry, pp. 256-287. W. H. Freeman and Company, San Francisco.

_____. 1969 b. Biometry, pp. 287-295. W. H. Freeman and Company, San Francisco.

Spackman, D. H., Skin, W. H., and Moore, S. 1956. Automatic recording apparatus for use in chromatography of amino acids. Federation Proceedings, 15, 358.

Spies, J. R., and Chambers, J. R. 1948. Chemical determination of tryptophan. Anal. Chem. 20, 30-39.

Spies, R. J. 1967. Determination of tryptophan in protein. Analytical Chemistry, Vol. 39, Nr. 12, 1412-1416.

Steel, B. F., Sauberlich, H. E., Reynolds, M. S., and Baumann, C. A. 1949. Media for Leuconostoc mesenteroides P-60 and Leuconostoc citrovorum 8081. J. Biol. Chem. 177, 534-544.

White, A., Handler P., and Smith, E. L. 1968. Principles of Biochemistry, p. 566. McGraw-Hill Book Company, N.Y., Sydney, Toronto, London.

Williams, H. H. 1959. Amino acid requirements. J. Americ. Dietet. Ass. 35, 929-933.

Watts, H. J., Booker, L. K., McAfee, J. W., Graham, D. C. W., and Jones, Jr. F. 1959. Biological availability of essential amino acids to human subjects. II. Whole egg, milk, and cottage cheese. J. Nutr. 67, 497-508.

APPENDICES

Appendix 1: Basal medium for S. zymogenes (1)

Glucose (g)	12
K_2HPO_4 (g)	18
Citric acid (g)	0.5
Sodium acetate (trihydrate) (g)	2.5
Tween 80 (ml)*	1
Solution of mineral salts (ml)**	10
Adenine (mg)	5
Guanine (mg)	5
Uracil (mg)	5
Xanthine (mg)	5
Thiamine (mg)	2
Pyridoxal ethylacetal hydrochloride (mg)	2
Riboflavine (mg)	2
Nicotin acid (mg)	2
Calcium pantothenate (mg)	2
p-Aminobenzoic acid (mg)	2
Folic acid (mg)	0.2
Biotin (µg)	10
Ascorbic acid (g)	0.5
Vitamin B_{12} (µg)	2

pH adjusted to 7.2 with acetic acid
Water added to 200 ml

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(1) Boyne et al. (1967).

*Polyoxyethylene sorbitan mono-oleate

**Contained $MgCl_2 .6H_2O$, 20g; $CaCl_2$, 5g; $FeCl_3 .6H_2O$, 0.5 g; $ZnSO_4 .7H_2O$, 0.5g; $MnSO_4 .4H_2O$, 0.25g; $CoCl_2 .6H_2O$, 0.25g; $CuSO_4 .5H_2O$, 0.25g; $VOSO_4$, 0.25g; Na_2MoO_4, 0.25g; dissolved in 1 l distilled water with addition of N-H_2SO_4 to clear.

Appendix 2: Amino acid supplement for S. zymogenes (1)

1-Glutamic acid (g)	1
1-Leucine (g)	0.5
1-Isoleucine* (g)	0.5
1-Valine (g)	0.5
1-Lysine hydrochloride (g)	0.5
1-Alanine (g)	0.5
1-Aspartic acid (g)	0.5
1-Arginine hydrochloride (g)	0.2
Glycine (g)	0.2
1-Cystine (g)**	0.2
1-Serine (g)	0.2
1-Tyrosine (g)	0.2
1-Proline (g)	0.2
1-Hystidine hydrochloride (g)	0.2
1-Phenylalanine (g)	0.2
1-Threonine (g)	0.2
1-Tryptophan (g)	0.2

pH adjusted with N-KOH to 7.2
Water added to 250 ml

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(1) Boyne et al. (1967).

*Allo free

**First dissolved separately in 10 ml boiling water by addition of HCl.

Appendix 3: Citrate cyanide buffer

Trisodium citrate (g)	5
Sodium cyanide (mg)	30

Dissolve in distilled water, adjust pH to 7.2 with N-H_3PO_4 and dilute to 1000 ml.

Appendix 4: Basal medium and amino acid supplement for *L. mesenteroides*, met assay (2)

	mg
dl-α-Alanine	200
l-Arginine HCl	242
l-Asparagine	400
l-Aspartic Acid	100
l-Cysteine	50
l-Cystine	
l-Glutamic acid	300
Glycine	100
l-Histidine HCl	62
dl-Isoleucine	250
dl-Leucine	250
l-Lysine HCl	250
*dl-Methionine	100
dl-Phenylalanine	100
l-Proline	100
dl-Serine	50
dl-Threonine	200
dl-Tryptophan	40
l-Tyrosine	100
dl-Valine	250
	g
Total weight	3.1
Glucose	25
Sodium acetate	20
Ammonium chloride	3
	mg
KH_2PO_4	600
K_2HPO_4	600
$MgSO_4 \cdot 7H_2O$	200
$FeSO_4 \cdot 7H_2O$	10
$MnSO_4 \cdot 4H_2O$	20
NaCl	10
Adenine sulfate . H_2O	10
Guanine . HCl . $2H_2O$	10
Uracil	10
Xanthine	10
Thiamine . HCl	0.5
Pyridoxine . HCl	1.0
Pyridoxamine . HCl	0.3

	mg
Ca dl-Pantothenate	0.5
Riboflavin	0.5
Nicotinic acid	1.0
p-Aminobenzoic acid	0.1
Biotin	0.001
Folic acid	0.01
Pyridoxal . HCl	0.3
	ml
Distilled H_2O to	500

(2) Steele et al. (1949).

*Omitted for 1-met assay

Appendix 5: Composition of the cystine assay medium for L. mesenteroides P-60 (3) (made up from prepared stock solutions)

Ingredients	Number of test tubes					
	100	125	150	175	200	300
Glucose	20g	25g	30g	35g	40g	60g
Na-Ac	12	15	18	21	24	20
NH_4-Cl	6	7.5	9	10.5	12	18
Peptone-H_2O_2 treated	150ml	187.5ml	225ml	262.5ml	300ml	450ml
dl-tryptophan 10mg/cc	10	12.5	15	17.5	20	30
l-tyrosine 2.5mg/cc	40	50	60	70	80	120
dl-methionine 10mg/cc	10	12.5	15	17.5	20	30
Adenine, Guanine, Uracil 1mg/cc	10	12.5	15	17.5	20	30
Thiamine 200 µg/cc	5	6.25	7.5	8.75	10	15
Pyridoxine 200 µg/cc	10	12.5	15	17.5	20	30
Ca-Pantothenate 50 µg/cc	40	50	60	70	80	120
Riboflavin 100 µg/cc	20	25	30	35	40	60
Nicotinic Acid 100 µg/cc	20	25	30	35	40	60
Biotin 0.5 µg/cc	10	12.5	15	17.5	20	30
Salts A&B As is	5	6.25	7.5	8.75	10	15
P-aminobenzoic acid As is	1	1.25	1.5	1.75	2	3
Folic Acid 1ml in 250	3	3.75	4.5	5.25	6	9
Dilute to	500	625	750	875	1000	1500 ml

(3) Received through the courtesy of Dr. R. J. Evans and Miss D. H. Bauer, Department of Biochemistry, Michigan State University, E. Lansing, Mich.

As indicated the amino acid supplement was partially replaced by hydrogen peroxide treated peptone prepared as described by Lyman et al. (1946):

Preparation of hydrogen peroxide-treated peptone: Fifty g of bacto-peptone in 500 ml of N-HCl were treated with 0.05 mol of hydrogen peroxide (5.7 g of 30% H_2O_2) and allowed to stand overnight at room temperature. The solution was then heated in a steam sterilizer at atmospheric pressure (100 C) for 30 minutes, cooled, neutralized with sodium hydroxide and steamed again, this time for one hour. The purpose of the second steaming was to decompose any hydrogen peroxide not used up by the oxidative reactions. The preparation was ready for use after diluting to a final volume of one liter.

Preparation of the stock solutions (Evans and Bauer, 1970)

dl-Methionine: 2.5 g dl-methionine was dissolved in 200 ml distilled water and warmed up on the steam bath. Before the solution was made up to a volume of 250 ml, 2 drops of conc. HCl were added.

dl-Tryptophan: 2.5 g dl-tryptophan was dissolved in 150 ml water with the aid of 5 drops conc. HCl and heated on the steam bath until completely dissolved. Then the solution was cooled to room temperature and made up to a volume of 250 ml.

1-Tyrosine: 1.25 g l-tyrosine was dissolved in 400 ml distilled H_2O, a few drops of 12% NaOH added and made up to a final volume of 500 ml.

Glucose, sodium acetate, and ammonium chloride were weighed out as required.

Adenine, guanine, uracil: 0.2 g of each was transferred into a 200 ml volumetric flask. The solution was warmed up on a steam bath and conc. HCl was added until the solution turned clear.

Nicotinic acid: A separate stock solution was prepared by dissolving 0.5 g niotic acid in 500 ml of distilled water. The stock solution was diluted one in ten.

P-aminobenzoic acid: 50 mg was dissolved in 500 ml distilled water.

Calcium-pantothenate: 50 mg was dissolved in 999 ml distilled water and 1 ml of salt solution A was added.

Pyridoxine: 50 mg was dissolved in 250 ml distilled water.

Riboflavine: 1 ml of glacial acetic acid was added to 50 mg anhydrous riboflavine and heated over the steam bath in 499 ml water.

Folic acid: 10 mg folic acid was dissolved in 10 ml distilled water containing a few drops 12% NaOH. The stock solution was diluted 1 in 250.

Biotin: 25 mg biotin was dissolved in 50 ml 80% ethanol. The stock solution was diluted 1 in 100.

Thiamine: 50 mg was dissolved in 250 ml distilled water.

Salt A: 25 g of each, K_2HPO_4 and KH_2PO_4 were dissolved in 250 ml distilled water containing a few drops of conc. HCl and conc. H_2SO_4.

Salt B: 10 mg $MgSO_4 \cdot 7H_2O$, 0.5g NaCl, 0.5g $MnSO_4 \cdot 2H_2O$, and 0.5g $FeSO_4 \cdot 7H_2O$ were dissolved in 250 ml distilled water containing a few drops conc. HCl.

All solutions described above were stored in the refrigerator.