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Development and Use of a Proficiency Test Specimen for Paralytic Shellfish Poisoning

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DEVELOPMENT AND USE OF A
PROFICIENCY TEST SPECIMEN FOR
PARALYTIC SHELLFISH POISONING

BY

JOHN A. ARES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
FOOD SCIENCE AND TECHNOLOGY, NUTRITION AND DIETETICS

UNIVERSITY OF RHODE ISLAND

1980

MASTER OF SCIENCE THESIS

OF

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1980

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ABSTRACT

Paralytic shellfish poisoning (PSP) is a serious, frequently fatal malady resulting from the ingestion of contaminated shellfish. Filter feeding bivalves, including clams and mussels, become toxic after ingesting toxic dinoflagellate algae of the genus Gonyaulax.

Due to strict guidelines on shellfish bed closures, thousands of dollars depend on an analyst's precision in measurement of the toxin. Measurement has traditionally been by means of a mouse bioassay. Since no proficiency test existed, the purpose of this work was to develop one.

Accordingly, after testing a number of substrates, including clams and mussels, a proficiency test specimen was formulated by the use of saxitoxin in a mashed potato matrix. These samples containing 200 and 500ug/100g saxitoxin dihydrochloride were sent to 16 state and Canadian laboratories for analysis in duplicate. The results revealed that within laboratory variance was less than between laboratory variance. The source of the errors was poor dilution technique by some laboratories. Estimates were found to be inflated due to initial overestimation of toxicity. Making solutions more toxic produced one of several "correct" dilutions that produced death times in the 5-7 minute range. It was also found that when using saxitoxin, circadian rhythm of the mice has no effect on their response.

The simplicity of the mashed potato matrix makes it a

suitable matrix for collaborative studies. Periodic use of this test specimen will help insure correct analysis and maximum utilization of shellfish resources.

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PREFACE

The thesis consists of a Manuscript on the development of a proficiency test for PSP. This manuscript was prepared according to the format specified by the Journal of Food Safety

Appendix I consists of a review of the literature.

Appendix II consists of the official method of analysis for PSP.

Appendix III consists of all references used in the thesis.

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MANUSCRIPT

**DEVELOPMENT AND USE OF A PROFICIENCY TEST
SPECIMEN FOR PARALYTIC SHELLFISH POISONING**

ABSTRACT

A proficiency test specimen was developed for evaluating laboratory precision in paralytic shellfish poisoning bioassays. Such a specimen is needed since much of health and economic consequence depends on an analyst's precision in measuring the toxin. Therefore, after testing clams, mussels, and mashed potato matrices, it was decided to use a test specimen composed of saxitoxin dihydrochloride and hydrated potato flakes. Samples at two dosage levels were sent to 16 collaborating laboratories. Analysis of the results identified errors arising from PCR dilution techniques. Within laboratory variance was less than between laboratory variance, and the variances were comparable to those obtained with naturally toxic clams. Since potato matrix offers many cost and convenience advantages, periodic use of the test specimen by private and regulatory laboratories will help insure precise analysis and maximum utilization of shellfish resources.

INTRODUCTION

Paralytic shellfish poisoning (PSP) has been a public health problem since 1793 (Prakash et al., 1971). During a 1972 red tide, losses of up to \$29 million dollars were attributed to PSP (Jensen, 1975).

The standard method of testing for the presence of PSP toxins in shellfish is a mouse bioassay (American Public Health Association, 1970). In use since 1937, the mouse assay, for means of simplicity and reliability has not been replaced by newer, chemical methodologies (Shimizu, 1979).

From the public health and economic viewpoints, it is essential that Federal and State laboratories be precise in their analysis of PSP, since shellfish beds must be closed to harvesting at levels of 80ug/100g shellfish meat (American Public Health Association, 1970). It is apparent that a proficiency test specimen should be used periodically to spot check for substandard laboratory technique in addition to the conversion factor check with saxitoxin dihydrochloride (STX) standards. Yet, there has been no means of testing laboratory's proficiency in PSP analysis. In fact, the last collaborative study involving PSP was conducted by McFarren (1959), but no detailed procedure was described for the preparation of the split samples.

The Microbiology task force assembled at the 10th National Shellfish Sanitation Workshop in 1977 recommended the development of such a test specimen (Hunt, 1978). In response to this need, this paper describes the development

of an artificially toxic matrix for use as a proficiency testing method. To prove the utility of such a test specimen, a collaborative study was performed with fourteen laboratories that regularly analyze shellfish for PSP.

MATERIALS AND METHODS

Selection of matrix

The purpose of the first phase was to determine a suitable carrier substance for STX, from which a homogenized split sample could be obtained. Four matrices were chosen for study: the soft shell clam, Mya arenaria; the blue mussel, Mytilus edulis; the bay quahog, Mercenaria mercenaria and instant mashed potato. The concept of using mashed potato in split sampling has been recognized for several years (Hunt, 1972).

Enough shellfish from each species were shucked to produce 600g of meats. Each sample of shellfish meat was blended at high speed until a uniform paste was produced. Shellfish paste (100g), was dispensed into each of five beakers and acidified with 100 ml of 0.18N HCl with stirring. The pH was adjusted to be between 3.0 and 3.5. Two vials of STX, each containing 500ug in 5.0 ml were diluted and brought to a volume of 25.0 ml with pH 3.5 HCl and stirred. Five ml portions were distributed into each flask and stirred. Thus, each matrix contained 200 ug of STX preceding extraction. The remaining procedure followed

the standard A.O.A.C. method for analysis of PSP (American Public Health Association, 1970).

Commercial instant mashed potato flakes were used for the preparation of the mashed potato matrix. Potato flakes (200g) were added to 800 ml of boiling distilled water, producing 1000 g of mashed potatoes. Then five, 100g portions were dispensed into 400 ml beakers and toxified using the method previously described for "preparation of toxic shellfish" with only one deviation. Since, after the extraction process, the liquid in the potato matrix did not settle out readily, a clinical centrifuge was used at high speed for ten minutes in order to produce a clear supernatant. The resulting liquid, along with the three prepared shellfish samples, were tested for toxicity by the mouse bioassay.

After analysis of the data from the first experiment, mashed potato was selected as the matrix for use in the collaborative study. Accordingly, in order to arrive at a thoroughly homogenized test specimen, the procedure was modified slightly from the method previously described. After the water had boiled, four 5.0 ml. vials of STX, each containing a total of 2,000 ug of toxin, were added to the 780 ml of water. The mixture was then stirred for several minutes. Then, 200 g of potato flakes were added and the mixture continually stirred until all flakes had become incorporated into the paste. The final preparation of potato contained STX at the level of 200ug/100g. For the

500ug/100g level, 5,000 ug of toxin was added to the water. All other procedures were identical to preparation of the 200ug/100g sample. This sample was used to test for homogeneity and percentage recovery.

Determination of shipping characteristics

An insulated package used for seawater split sampling by the FDA, Northeast Technical Services Unit was tested for suitability for use in the collaborative study. Two sample containers, along with two ice packs and a vial containing 15 ml of 1.0 ug/ml STX standard were included in the package. Two packages were prepared, one containing refrigerated samples, and the other contained frozen samples. Before storage, thermocouples were placed into the center of the matrices and the vial of STX. The ice packs were frozen and packed in the boxes prior to addition of the cold sample bottles. After 33 days of cold storage, the frozen and refrigerated samples were allowed to warm at room temperature. The warming cycle was monitored using a Honeywell multi point temperature recorder.

Conduct of the collaborative study

Fifteen laboratories that regularly perform PSP analysis participated in the study. These laboratories included, the Massachusetts State Department of Health laboratories at Amherst, Fairhaven, Jamaica Plain and the Lawrence Experimental Station; Maine Dept. of Marine Resources

Laboratory, Boothbay Harbor ; The Dept. of Health, Hartford, Conn. ; National Health and Welfare , Ottawa, Canada ; The State Consumer Protection Laboratory , Concord, N.H. ; The New York City Dept. of Health, N.Y. ; Rhode Island Dept. of Health, Providence, R.I. ; Purdue University Dept. of Microbiology ; California State Dept of Health, Berkeley, Ca. ; Public Health Laboratory, Portland, Or. ; Alaska State Dept of Health, Juneau, Al. ; Northeast Technical Services Unit, Davisville, R.I. ; Wash. Dept. of Social and Health Services, Seattle, Wash.

Instructions describing the the procedures were sent prior to the actual mailing of samples, to assure availability of mice and materials. The samples were sent by Federal express or U.S. Post Office express mail, depending on the distance from Rhode Island. The sample designated for the R.I. State Dept. of Health was hand delivered a month later , after continuous refrigeration.

With the availability of a new batch of crystalline STX, containing approximately 10,000ug of toxin, the toxic mashed potato matrix was prepared as previously described with the following alterations. Toxicity testing of the crystalline STX, diluted with 100ml of pH 3.5 HCl indicated a total value of 110 ug/ml Thus, 18.0 ml. of the above contained 2000 ug of STX. When added to 780 ml of water and 200g of potato flakes, this produced 1000 g of toxified mashed potatoes. The final concentration of the test sample was 200ug/100g. For the 500 ug. level, 45 ml. of toxin solution

were added to 755 ml of water to produce 1000 g of toxic mashed potato containing 500ug/100g of STX. Then 250 g of the homogenous toxic products were dispensed into 16 bottles for each level and, immediately refrigerated at 35 degrees F. A total of 32 bottles were prepared for both levels of STX.

Two 250g samples of toxic mashed potatoes were enclosed in an insulated shipping carton. One bottle was labeled A and the other B. Level A was the 200 ug sample, and level B was the 500 ug sample. The laboratories had no prior knowledge of the levels. The collaborators were simply instructed to report the toxicity of the samples using their routine procedure. Duplicate samples were to be run on each bottle. Four toxicity values were to be reported. The package also included a vial containing 15 ml of 1.0 ug/ml of STX standard which was designated for use in the periodic check of conversion factors (C.F.). An envelope was included with instructions that explained the centrifugation step and how the data should be calculated and reported. A franked, addressed envelope was sent with the package to expedite return mailing of results.

Effect of circadian rhythm

In order to determine if circadian rhythm of the mice was responsible for contributing any amount of error, six mice were injected with 1.0 ml of 0.33ug/ml STX every four hours during a 24 hour period. Seven sets of mice were

injected using a total of 42 mice. Temperature in the mouse room was held at 20 degrees C. and lighting was continuous. Food and water were provided ad libitum. Care was taken to avoid traumatizing the mice prior to injection.

Statistical analysis

The statistical techniques used, were modifications of procedures outlined in Youden and Steiner's (1975) manual of statistical techniques for collaborative tests. Homogeneity of samples was determined by performing a t-test on the difference between means of replicate samples containing either the 200 or 500 ug/100g level of STX. Total variance, S_d was considered as being the sum of within laboratory variance (S_r^2) and between laboratory variance (S_b^2). It was calculated by squaring the standard deviation of all determinations of a particular level of toxin. Precision error was calculated as $S_r = \sqrt{\frac{\sum d^2}{2n}}$ where d is the difference between replicates. S_r is considered to be a measure of repeatability or within laboratory error. Reproducibility, a measure of the errors which may be encountered between different laboratories, was denoted by S_b . The notation S_b , the between laboratory variance, was calculated using: $S_d^2 - S_r^2 = S_b^2$. Outliers were determined by procedures described in the National Bureau of Standards Handbook No. 91 (Natrella, 1963).

RESULTS

The results of the first phase are reported in table 1. With all five replicates considered, a higher mean STX recovery value and coefficient of variation were obtained for Mya arenaria. When the value for the third replicate of Mya arenaria was eliminated as an outlier, the new recovery mean became 102ug/100g with a coefficient of variation similar to Mytilus edulis (10.0). Thus, while Mya arenaria yielded the highest recovery, the coefficient of variation was least with mashed potatoes (6.0).

Table 2 shows the results of using two different methods of toxifying mashed potato matrix. Comparison of the means using a t-test showed no difference between adding pre-measured toxin to each beaker of mashed potato or toxifying the water before addition of potato flakes. However, the water toxification procedure was the practical way to make large quantities of homogenous samples for shipment to collaborators.

Toxified clam homogenate was compared with toxic mashed potatoes in order to confirm the suitability of the potato matrix at high toxin levels. The results of testing the 500ug/100g toxicity level are shown in Table 3. Mean recoveries for the clam homogenate and mashed potato were very similar, but the standard deviation and coefficient of variation were lower for the mashed potato matrix.

Of the 16 laboratories sent the toxified mashed potato samples, two did not analyze the samples after receiving

them. Laboratory 12 was the only collaborator which did not report results in duplicate. Table 4 presents the reported low level (200ug/100g) values reported by 14 laboratories. The rejection range for outliers in the low level sample was <34 and $>122\text{ug}/100\text{g}$. On this basis, laboratories 9 and 11, had values outside this range. Table 5 shows the collaborator's results of analyses of the 500ug/100g samples. The rejection range for the outliers at this level was <161 and $>542\text{ug}/100\text{g}$. Laboratories 9 and 10 were outliers on the high side while laboratory 3 had one outlier on the low end.

The means for all reported high and low levels of STX by the 14 collaborating laboratories are given in Table 6. When these data were subjected to an analysis by Duncan's multiple range test, laboratories 9 and 10 were isolated as being statistically different at the high recovery end, and laboratory 3 was found to be significantly different at the low recovery end. Laboratory 11 was not found to be statistically isolated, since it was grouped with those laboratories ranked in the middle of the array.

In searching for possible causes of variation, the circadian rhythm of the mouse was considered. As shown in figure 1, no difference in mouse response was obtained during any particular time of day. Response to STX was fairly uniform, and the variation produced was attributed to the variation inherent in the mouse test itself.

Table 1. Recovery of toxicity from four matrices.
Saxitoxin dihydrochloride added at 200ug/100g

Replicate	<u>Mercenaria</u> <u>mercenaria</u>	<u>Mya</u> <u>arenaria</u>	<u>Mytilus</u> <u>edulis</u>	Instant mashed potato
	ug/100g	ug/100g	ug/100g	ug/100g
1	64	110	88	60
2	61	101	86	63
3	79	(153) ¹	74	58
4	56	94	76	61
5	79	102	70	67
Mean	67.8	102.0	78.8	61.8
Standard deviation	10.6	10.1	7.8	3.4
Coefficient of variation	0.16	0.09	0.10	0.06
Percentage recovery of toxicity	33.9	51.0	39.4	30.9

¹ Eliminated as an outlier

Table 2. Recovery of toxicity using two methods of toxification. Saxitoxin dihydrochloride added at 200ug/100g

Replicate	Toxin dispensed into mixed potato matrix	Toxin dispensed into water before addition of flakes
	ug/100g	ug/100g
1	60	63
2	63	70
3	58	59
4	61	75
5	67	62
6	55	79
7	63	63
8	62	71
9	64	82
10	77	65
11	-	64
Mean	63.0	68.5
Standard deviation	5.6	7.2
Coefficient of variation	0.09	0.11
Percentage recovery of toxicity	31.5%	34.3%

Table 3. Recovery of toxicity from two matrices.
Saxitoxin dihydrochloride added at 500ug/100g

Replicate	<u>Mercenaria</u> <u>mercenaria</u>	Mashed potato
	ug/100g	ug/100g
1	315	370
2	326	312
3	335	387
4	376	374
5	334	338
6	442	314
7	308	-
Mean	348	349
Standard deviation	43.4	29.5
Coefficient of variation	0.13	0.09
Recovery percentage of toxicity	69.6	69.8

Table 4. Saxitoxin levels reported by collaborating laboratories assaying duplicate mashed potato matrix samples, each containing 200ug/100g added toxin

Laboratory	First replicate		Second replicate	
	Toxin	Dilution factor	Toxin	Dilution factor
	ug/100g		ug/100g	
1	70	1.0	76	1.0
2	108	1.6	100	1.6
3	68	1.0	66	1.0
4	80	1.0	78	1.0
5	74	1.0	74	1.0
6	70	1.0	74	1.0
7	98	1.5	95	1.5
8	76	1.0	65	1.0
9	(154) ¹	2.8	(190)	3.5
10	91	1.2	108	1.2
11	(127)	2.0	(135)	2.0
12	67	1.0	-	-
13	57	1.0	60	1.0
14	73	1.0	65	1.0
Mean	86.7		91.2	
Standard deviation	25.9		41.5	
Coefficient of variation	0.29		0.45	
Recovery percentage of toxicity	44.0%		46.0%	
Overall mean		88.8		
Overall Standard deviation		31.4		
Overall Coefficient of variation		0.35		

¹ Numbers within parentheses designated as outliers

Table 5. Saxitoxin levels reported by collaborating laboratories assaying duplicate mashed potato matrix samples containing 500ug/100g added toxin

Laboratory	First replicate		Second replicate	
	Toxin	Dilution factor	Toxin	Dilution factor
	ug/100g		ug/100g	
1	400	5.0	387	5.0
2	441	6.0	380	7.5
3	(157) ¹	3.0	176	3.0
4	386	4.5	345	4.5
5	291	3.0	283	3.0
6	306	4.0	287	4.0
7	338	5.0	336	5.0
8	412	5.0	395	5.0
9	(550)	12.5	(594)	11.0
10	(546)	7.0	508	7.0
11	357	5.0	426	5.0
12	265	3.5	-	-
13	253	4.0	312	6.0
14	396	5.0	403	5.0
Mean	364		371	
Standard deviation	104.5		100.6	
Coefficient of variation	0.28		0.27	
Recovery percentage of toxicity	73.0%		75.0%	
Overall mean		367		
Overall Standard deviation		102.5		
Overall Coefficient of variation		0.28		

¹ Numbers within parentheses designated as outliers

Table 6. Mean of all reported saxitoxin levels and ranking of collaborating laboratories by Duncan's multiple range test.¹

Grouping ²	Mean of all values ug/100g	Collaborating laboratory
A	372	9
B	313	10
C	261	11
C	257	2
C D	237	8
C D	234	14
C D	233	1
D	222	4
D	216	7
E	184	6
E	180	5
E	170	13
E	166	12
F	116	3

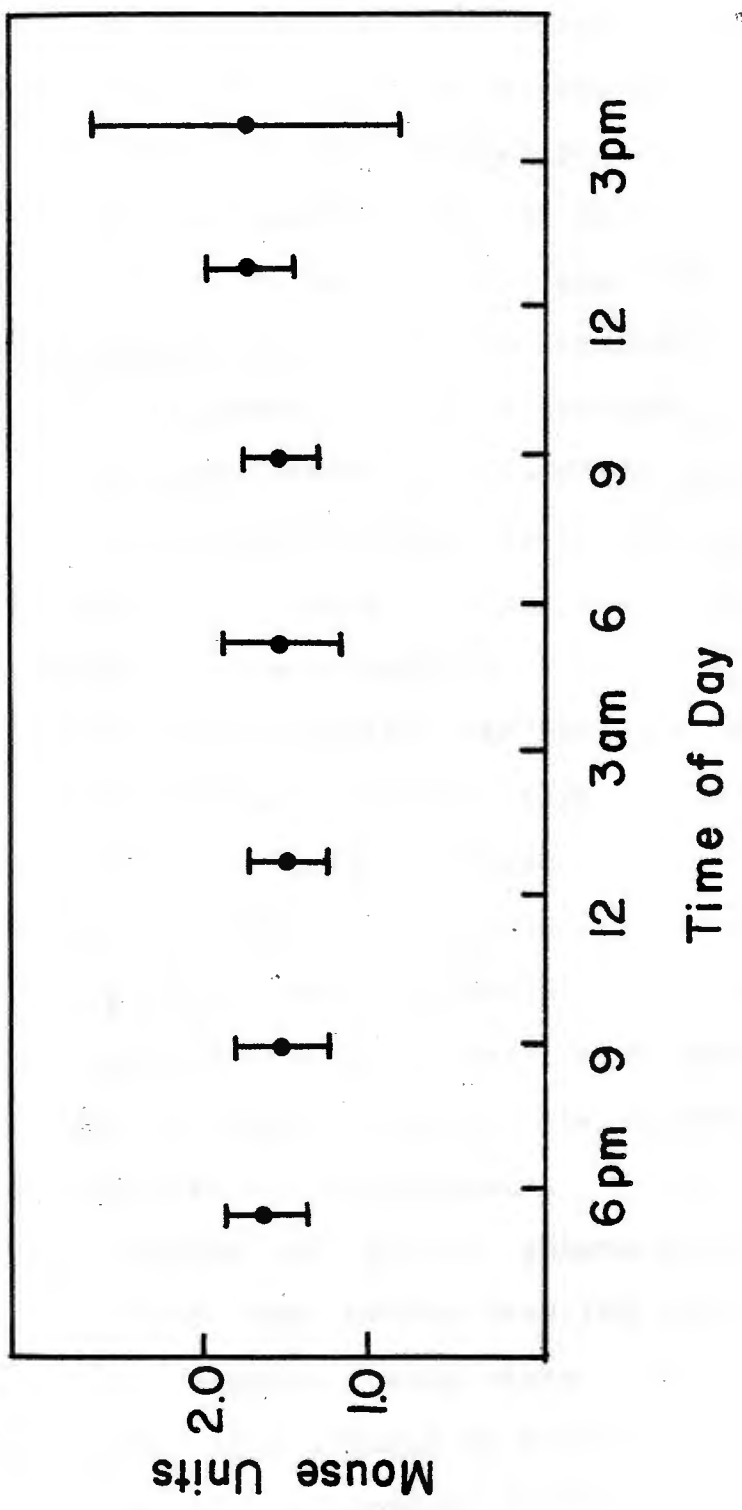
¹ Means represent four values except laboratory 12 which reported only one high value and one low value

² Means with the same letter are not significantly different

FIGURE 1

EFFECT OF CIRCADIAN RHYTHM ON MICE
INJECTED WITH 0.33UG/ML SAXITOXIN DIHYDROCHLORIDE

Each point represents mean corrected
mouse unit for six mice and
95% confidence limits



DISCUSSION

The data presented in Table 1 shows that washed potato or any of the shellfish matrices could serve as a carrier for pure STX and be used in a collaborative study. Percentage recovery of STX ranged from 31 to 51% for the 200ug/100g level of inoculation. At the 500ug/100g level, percentage recovery of added STX was 70% for both Mercenaria mercenaria and washed potatoes (Table 2.). This difference was expected. The physiological salt effect observed by McFarren (1959) was expected, but this may not explain the low yields. Other reactions may be occurring that could bind the toxin, but they did not prevent consistent recovery percentages.

Other than always having the smallest coefficient of variation, the washed potatoes offered several unique advantages over the shellfish matrices.

With clams or other shellfish, the meats must be shucked, blended and then carefully toxified. This procedure is rather time consuming, messy and tedious. In the preparation of washed potato, the simple procedure as outlined assures complete homogeneity.

Since the supply of washed potato is not seasonally dependent, it does not suffer from the logistic problems associated with shipping toxic clams. Since clams do not have to be dredged, this results in a cost reduction as well as the elimination of a biological variable.

As long as the potato is not frozen, texture and

toxicity remain stable. In the collaborative study, precise results were obtained up to two months after shipping. When frozen and thawed, there was a drip loss at the bottom of the sample container, and toxicity of the product was no longer found to be homogenous. Because of this, a refrigerated sample was used in the collaborative study.

A number of outlying values and laboratories are shown in Tables 4, 5 and 6. After elimination of outliers and recalculation the new averages were seen as 78.0 and 342 ug/100g. After examination of the original data presented by the laboratories that reported the outliers, a pattern became apparent in the method used at arriving at an appropriate dilution factor (DF). For Laboratory 10, their 500 ug/100g level value of 546 was classified as an outlier. In making their initial analyses, short death times were noted with three mice at a DF of 3.25. Therefore, a dilution was made to yield a factor of 7, which resulted in toxicities of over 500ug/100g. Our results indicated that a DF of 5 yielded death times in the 5-7 minute range using a conversion factor of 0.22. The laboratory used the 0.25 periodic check value instead of 0.24 which was their pre-determined conversion factor. While this would not cause the degree of deviation seen, it was a transgression of established procedure. Had dilutions been made in smaller, stepwise increments, a more conservative estimate of the toxin level would have been obtained. This was confirmed in a study conducted among personnel at the FDA,

Northeast Technical Services Unit . Those who were familiar with the bioassay were asked to analyze an extracted sample according to routine procedure. As shown in table 7, the method of dilution in a stepwise manner was critical. Laboratory 9 arrived at abnormally high results for their 200ug/100g samples using dilution factors of 2.75 and 3.5. These values were double the mean.

In similar fashion for the 500ug/100g level, Laboratory 9 reported results based on a dilution factor of 11 and 12.5. With this DF, proportionally smaller differences in death times occur. Thus, three or four dilution factors might produce 5-7 minute death times. Of these, the most conservative times should be chosen as correct. Otherwise, toxicity will be viewed as artificially high. Laboratory 11 had a DF of 2 for the low sample. Obtaining this factor appears to be the result of making solutions more concentrated until death times appeared in the 5-7 minute range . The high results obtained by Laboratory 9 and Laboratory 10 supports the process of making dilutions by a stepwise progression, starting with one. Laboratory 3, on the other hand, produced the only outlier on the low side for either sample level . Calculations and methods of dilution appear correct, however, the cause is unknown.

Previously, it had been assumed that time of day of mouse injection contributed to variation in death times (Prakash, et al. 1971). Halberg (1960), using endotoxin from E. coli found that percentage of death in mice varied

the same trends and increased with an increase in the level of toxin. For both levels measured by the collaborators, the mashed potato showed better precision and repeatability on duplicate samples than the spiked clam used by McFarren. Even when between laboratory error is considered, Figure 3 depicts mashed potato comparing favorably with McFarren's clam samples. This indicates that the reproducibility between laboratories was similar. Expressed as a total variance, mashed potato and McFarren's naturally toxic and spiked shellfish show nearly exact performance (figure 4).

On the basis of overall performance, it may be concluded that mashed potato is as good as natural or spiked shellfish for use as a proficiency test specimen. Since it offers some significant advantages in preparation, it is a satisfactory matrix for laboratory and collaborative studies.

Table 7. Preliminary split sample performed by three analysts within one laboratory using toxic mashed potato inoculated at 500ug/100g showing the importance of stepwise dilutions

Analyst	Measured STX level ug/100g	Death time	Corrected mouse unit	Dilution factor
A	374	5:45	1.74	5
		5:40	1.69	5
		6:00	1.70	5
B	370	5:36	1.69	5
		5:59	1.68	5
		7:01	1.39	5
C	489	6:05	1.60	8
		8:21	1.22	8
		7:00	1.39	8
C'	402	5:40	1.86	5
		7:05	1.39	5
		5:15	1.83	5

C' is analyst C given solution with a dilution factor of 5

FIGURE II

**COMPARISON OF PRECISION ERRORS
(WITHIN LABORATORY ERRORS)
USING MASHED POTATO AND
SPIKED CLAM MATRICES
AT DIFFERENT TOXICITY LEVELS**

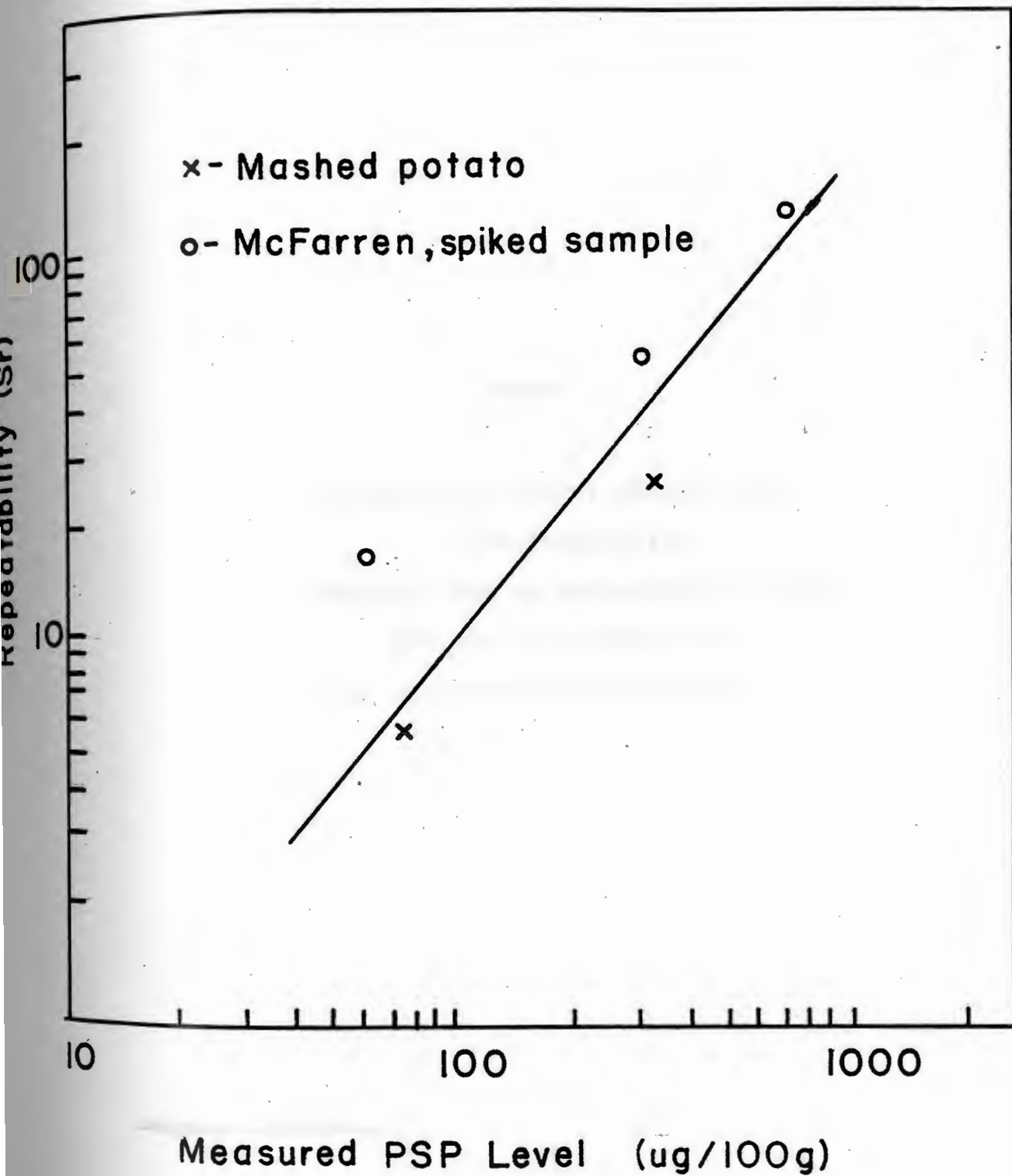


FIGURE III

**BETWEEN LABORATORY VARIABILITY
(REPRODUCIBILITY)
COMPARING USE OF MASHED POTATO WITH
MCFARREN'S SPIKED CLAMS
AT DIFFERENT TOXICITY LEVELS**

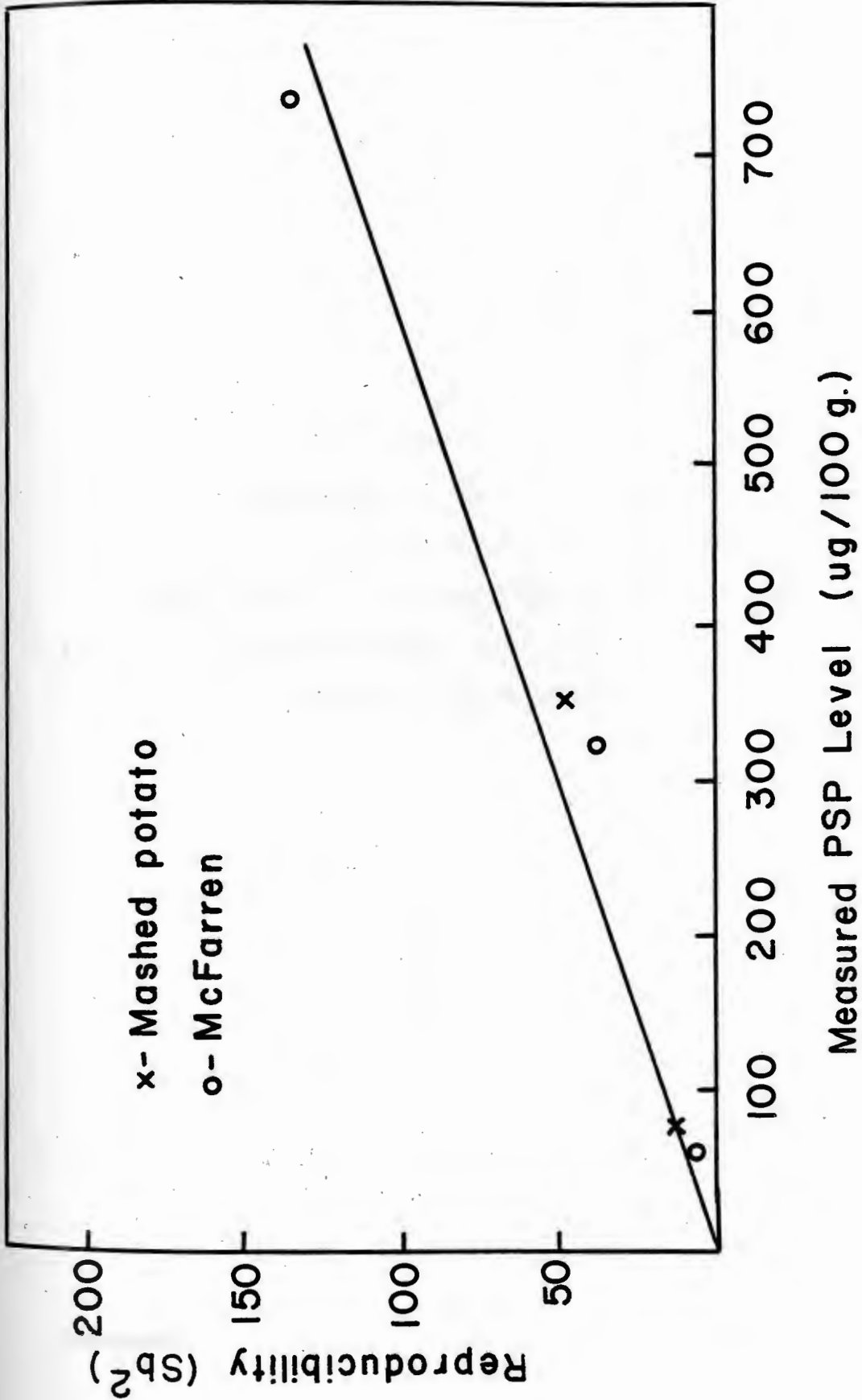
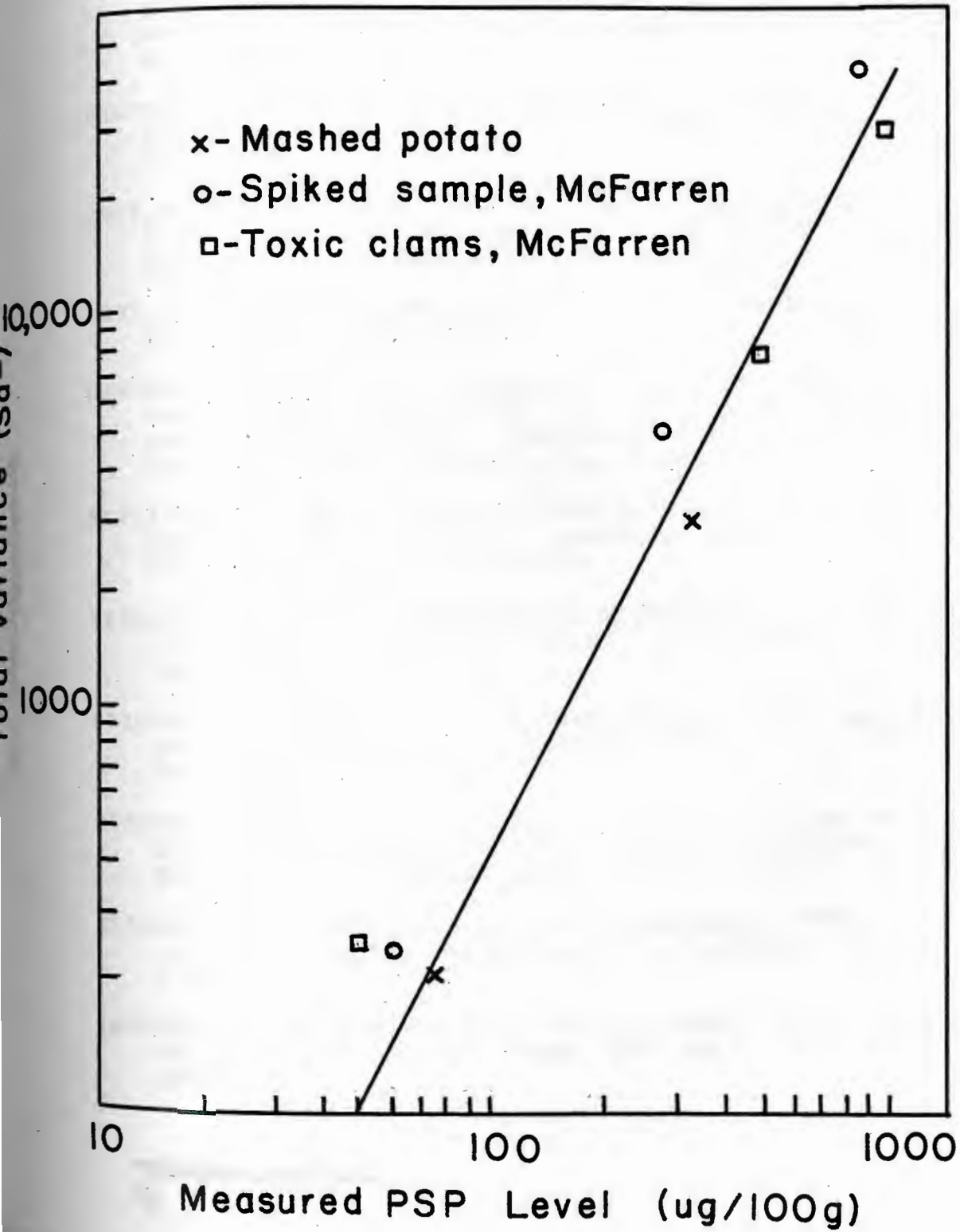


FIGURE IV
COMPARISON OF TOTAL VARIANCE
OF THREE COLLABORATIVE STUDIES USING
MASHED POTATOS, TOXIFIED CLAM HOMOGENATE AND
NATURALLY TOXIC CLAM HOMOGENATE
AT DIFFERENT TOXICITY LEVELS



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Characterization of the toxin

paralytic shellfish poisoning (PSP) results from the ingestion of shellfish contaminated by a complex of dinoflagellate toxins. Members of the genus *Gonyaulax* produce poisons but the taxonomy of the species has not been fully clarified. (Alam et al., 1975) *Gonyaulax catanella* and *G. tamarensis* have been responsible for outbreaks on the west and east coasts of North America, respectively. The potency of *G. tamarensis* is not consistent. Strains of varying toxicity have been found (Alam et al., 1979). These also varied morphologically, containing toxic and non-toxic varieties (Loeblich and Loeblich, 1975). Loeblich (1978) found three of 10 toxic strains of *G. tamarensis* var. *excavata* to be non-bioluminescent

Although no outbreak has ever been reported in fresh water, Jackim and Gentile (1968) reported the presence of a toxin similar to STX (stx) in the alga

Aphanizomenon flos-aquae. Reactions of mice are the same as STX although the lethal dose appears to be somewhat greater.

Considerable progress has been made in the characterization of the toxin. Until 1974, it was assumed that STX was the only substance responsible for PSP. However, by that year it became apparent that at least two substances were causing toxicities on the east coast, and evidence was presented suggesting a third (Shantz et al., 1975; Shimizu, et al. 1975b ;. ; Buckley, et al. 1975). Identification of four toxins was reported a year later by

shimizu et al. (1975a). These included STX (STX), and three others called gonyautoxin I (GTX-1), gonyautoxin II (GTX-2) and gonyautoxin III (GTX-3). These were isolated from cultures of G. tamarensis cells.

Six toxins were separated from Japanese shellfish by Oshima et al. (1976). G. catanella was implicated as being the source of the toxins in the shellfish. The two new toxins were named GTX-1' and JGTX-1 (Oshima, et al. 1978). These toxins were found to be the same as those from G. tamarensis cells (Oshima, et al. 1977).

The proportion of the toxins in relation to each other varies with species. The Japanese mussels, made toxic by G. catanella, contained higher amounts of GTX-1 and GTX-5 than fractions isolated from G. tamarensis cells (Oshima, et al. 1976). In fact, STX, which has always been considered to be the major poison responsible for PSP, is the dominant toxin in the butter clam (Shimizu, et al. 1977). Shimizu et al. (1977) also reported that GTX-1 and GTX-2 appear to contribute the majority of toxin in most species of shellfish analyzed. Oshima and co-workers, (1977) reported the presence of another new toxin from butter clams which was called neOSTX (neOSTX). This was the first account of a toxin other than STX being present in these clams. NeOSTX has also been isolated from G. tamarensis cells, bringing the known number of toxins to seven. They can all be separated by electrophoresis (Fallon and Shimizu, 1977). In 1977, Buckley et al. described

another means of differentiating toxins through the use of a PSP analyzer . Its operation is based on the measurement of the fluorescent products that the toxins yield under oxidative conditions. The analyzer scarcely detects neoSTX and thus requires the mouse test or thin layer chromatography to give an index of complete toxicity. Even though the analyzer elutes all of the toxins, GTX-1 gave a weak response to neoSTX (Oshima, 1979).

Testing methods

The first published report of a relationship between concentration of PSP and death time of a mouse appeared in 1932 (Frinzmetal et al. 1932) However, the source of the so-called "mussel poison" was still unknown. An average lethal dose was defined as "that amount of poison which will kill a mouse on intraperitoneal injection after 10 to 20 minutes." This became known as the mouse unit. Five years later, Schmeer et al. (1937), demonstrated the presence of PSP in Gonyaulax cells. Following this, Schmeer and Meyer (1937) described a quantitative mouse bioassay for PSP in which a dose response curve was constructed. Weight corrections for mice varying from the standard 20.0 g. were also included. These are currently known as the " Schmeers tables". For extraction of the toxin, acidified methanol was found to be effective. It was assumed that one mouse unit was equivalent to one microgram of "pure poison".

The original mouse assay remained essentially unchanged

until Stephenson et al. (1955) examined the test more critically. They found that mice in excess of 20g. did not show a linear response to the toxin. Accordingly, an additional factor of 1.6% was applied to the weight correction for each gram over 20.0. Injection of over 1500 mice showed that female mice were approximately 10% more sensitive to the toxin than were male mice of equal weight.

The next major development came two years later with the advent of an efficient procedure for isolation of the poison from toxic shellfish (Shantz, et al. 1957). Extraction of tissues with acidified aqueous ethanol was followed by an ion exchange treatment and chromatography of the fractions. Toxicity was estimated at 5500 mouse units/mg. In 1958, Shantz and associates published a procedure using the purified poison for standardization of the bioassay. This consisted of dilution of the calibrated vials of known toxicity and injection of mice to determine a conversion factor (CF) which equated mouse units (MU) to micrograms of toxin. These workers found that mice larger than 23.0 g. should not be used due to the high survival rate when low doses were administered. They also found that injection of three mice gave results that were statistically similar to assays using 10 mice.

Occasionally, mice do not die after intraperitoneal (IP) injection. Steward et al. (1968) found that there was a 14% failure rate inherent in the technique. Apparently, the toxin is absorbed and metabolized at a sub lethal rate. Kao

(1966) found that younger animals were more susceptible to the toxins than were the adults of the same species.

Specific treatment of the clams prior to analysis is not described in detail in the official method of PSP analysis (American Public Health Association, 1970). It has been proposed that shellfish should be refrigerated (not frozen) and analyzed within 6-24 hours after collection. (Meiscier and Adams, 1976).

In testing for recovery of added toxin it was determined that physiological salt was preventing full recovery from non toxic clams that had been "spiked" with STX. At levels of 100ug/100g. shellfish meat, only 40 to 60% recoveries were observed. The lack of sensitivity at these levels was deemed to be "the most undesirable feature of the bioassay". The percentage recovery increases towards 90% as the level of toxicity approaches 800ug/100g (McFarren, 1959). However, accurate measurement is most needed at the 80ug/100g. level. This is the limit considered safe for human consumption as determined by FDA (Clem, 1975). The Canadians had been using a value of 64 ug/100g. but increased the limit to coincide with the United States standard (Bond and Medcof, 1958).

The mouse bioassay, in its present form, has several disadvantages which support the search for alternative testing procedures. First, there is the need to maintain a mouse colony. Additionally, mice must be within the acceptable weight range. The limits of sensitivity are

dependent on the string of mouse used. The test is complicated by the fact that death time is somewhat subjective. Since death time is not linear with toxic dose, death times must be in the 5-7 minute range. Lastly, the mouse assay may underestimate marginally toxic clams by as much as 60% (Shimizu, 1979).

The outstanding advantage of the bioassay is that it gives a clear indication of total toxicity. The mice are sensitive to all toxins involved. Additionally, the test is relatively simple to perform.

Chemical testing procedures

Research has been directed towards development of a suitable chemical test. An assay that was sensitive at low levels was sought. McFarren et al. (1958) reported a method that detected PSP in clams by chemical determination. However, it offered no improvement at critical levels near 100ug/100g. The test suffered from a lack of specificity, and results were directly affected by the freshness of the clam. Removing the interfering substances required careful temperature control and spectral analysis. Since it was more complex and time consuming it offered no significant advantages over the mouse test. The chemical procedure was modified a year later (McFarren, et al. 1959) to give a twofold increase in sensitivity. However, it was not applicable to all shellfish. Specifically, the test was not successful in measuring toxicity in pacific oysters, due to

the presence of interfering material.

A third approach to PSP analysis was attempted when Johnson and Mulbery (1966) devised a hemeagglutination, serological assay. It was considerably more sensitive than the mouse bioassay, but it utilized an unstable sheep blood preparation. PSP toxin was also adsorbed to bentonite particles. This test was stable, but did not show an increase in sensitivity. Since reagent preparation was more complex than the bioassay, it was never adopted (Prakash, et al., 1971).

Bates and Rappaport (1975) developed a fluorometric assay that was specific for STX. According to the authors, the lower limit of sensitivity was 0.004ug/ml. However, being specific for STX alone was a significant disadvantage, since it is now known that STX is only one among several toxins. In the majority of cases, it does not represent the major portion of toxicity. In one report, STX was absent completely from naturally toxic Alaskan mussels (Shimizu, et al. 1978). Relying on a test specific for an absent toxin could prove to be disastrous. In addition to limited sensitivity, the assay also required strict adherence to protocol (Gershey, et al. 1977).

Gershey et al. (1977) also described a method that was specific for STX, but it was inferior to the Bates and Rappaport (1975) method. Guanidine containing compounds were potential sources of contamination. Additionally, six hours were required for the completion of a single analysis.

White and Maranda (1977) compared the Bates and Rappaport (1975) test with the bioassay and found that the chemical test recorded only 16 to 48% of the total toxicity found by the mouse bioassay. This variability would not lend its use to assay critical samples.

Clam characteristics

Certain species of filter feeding bivalves have been implicated as greater PSP risks than others. Almost two-thirds of all east coast PSP deaths have been caused by mussels. Most of the remaining one-third of PSP deaths were caused by soft shell clams. A few remaining deaths were caused by toxin from rough whelks and surf clams. Species that are not considered to be PSP hazards include bay quahogs, the ocean quahog and eastern razor clams. Hazardous west coast species include butter clams, the California mussel, the blue mussel and razor clams (Prakash, et al. 1971).

The degree of toxicity in shellfish depends on a number of factors other than the presence of toxic dinoflagellates. Medcof et al. (1947) reported that clams higher on the beach show less toxicity than those at the low water mark. This would seem plausible, since longer immersion times would allow a larger number of organisms to be filtered. Toxicity is also found to be higher at the mouths of bays and rivers. Presumably this is due to the lack of dilution or flushing with fresh water (Medcof, et al. 1947). Gonyaulax cells

isolated from three different bays during the same bloom were found to contain different toxicities for equivalent cell concentrations. Apparently, there are different strains of *Gonyaulax* with varying toxicity. (Alam, et al., 1979).

The average duration for a period of PSP toxicity is 34 days for mussels and 23 days for soft shell clams. However, mussels reach a mean toxicity of 580ug/100g. while soft shell clams average 290ug/100g. The depuration rates for mussels and soft shell clams are comparatively short in comparison to those of the surf clam, which may remain toxic for a year after a bloom (Medcof, et al. 1947). Butter clams will retain toxicity for up to two years (Lutz, 1977). This may be due in part to the presence of melanin in the clam which may impart a protective action (Price and Lee, 1972b). The association of melanin and PSP is reversible and may be broken down by the addition of cations to the water. Bound PSP decreases by 68% in the presence of Al^{+++} (Price and Lee, 1972a). The butter clam may have an advantage over other shellfish. PSP may greatly reduce the filtration of water in species such as soft shell clams (Gilfillan and Hanson, 1975)

Although the only reported cases of PSP have resulted from toxic mollusks, it is possible to transmit the toxin through the food chain (Foxall, et al. 1979). They have shown that crabs can accumulate PSP after 15 weeks on a diet of toxic soft shell clams. The highest toxin levels were

not considered a health hazard , but did raise questions of the extent that PSP toxin travels in the food chain.

Ozone detoxification

Contamination of shellfish in locations such as Alaska and the Bay of Fundy renders potentially valuable resources virtually useless due to long depuration times. Butter clams in Alaska remain toxic during much of the year making exploitation of the resource impractical (U.S. Dept. of Commerce, 1977).

Three methods have shown varying degrees of success in detoxifying shellfish of PSP. The French have used chlorination for reducing toxicity but it produces a poor flavor. Thermal shock produces a lowering of PSP toxicity but not to the magnitude seen with ozonation. Treatment with ozone is the most viable alternative , but it is not currently cost effective (Blogoslawski, 1979). Ozone has been used since 1929 to sterilize seawater in shellfish depuration stations and in marine labs dependent on a non-toxic seawater supply (Blogoslawski, et al., 1975). Ozone has also been used for the inactivation of the toxin from C. botulinum (Blogoslawski, et al., 1973).

Surf clams show long retention times of PSP. They may hold the toxin up to three months after relaying to cleaner waters (Blogoslawski and Stewart, 1978). However, Medcof et al. (1947) found ozone to be effective in reducing PSP toxicity in surf clams. The ozone procedure depurates the

organs of the surf clam at varying rates . The foot is the only tissue that will depurate to safe levels within 14 days. Since ozone depuration has met with varying success, more research is needed before the procedure can be utilized on a large scale. Blogoslawski et al. (1975) used ozone on the toxin from G. breve and achieved success in lowering the potency of the toxin. In 1976, Dawson et al. reported detoxification of contaminated mussels (2586ug/100g.) in eight days using ozonated seawater. The implications of long-term use of ozone with aquatic organisms is not fully understood. . Cases of abnormal larval development and tissue damage may have resulted from ozone and ozone by-products (Dawson,et al. 1976).

Economic halo

The consequences of a red tide are not always restricted to the immediate area of an outbreak. Seafood markets in adjoining states may be severely affected by bad publicity , even though no health threat is present. Public overreaction to a Massachusetts red tide in 1972 has been fairly well documented. Loss to fishermen was estimated by the National Fisheries Service to be more than \$1 million because of bad publicity (Jensen , 1975) Losses incurred from a 1974 bloom in Maine were an estimated \$10 to 36 million on the retail level. The hardship to clamdiggers and their families was estimated at \$1 to 2 million . This may be contrasted to a 1975 figure that quoted \$29 million

lost in destroyed clam resources as a result of all closures of shellfish beds. These include effect of sewage and pollution (U.S. Dept. Comm., 1977).

During the Massachusetts bloom of September 1972, all shellfish shipments were stopped from entering New York and Connecticut. This followed the Maine and Massachusetts ban on the harvest and sale of soft shell clams and mussels. Bay scallops were only briefly included in the ban. However, Atlantic oysters, unlike their pacific counterparts, have never been implicated in PSP outbreaks (Prakash, et al., 1971). They can accumulate the toxin if exposed to it, but they are not found in waters that experience toxic blooms. As a result of the economic halo, purchases of oysters declined (U.S. Dept. Commerce, 1977).

Oysters were not the only "innocent" species affected. Consumers also avoided fish, lobsters, sea scallops, and northern shrimp. As a result, lobstermen were unable to sell their catch, and the wholesale shellfish and finfish business decreased 25 to 50. Additionally, seafood restaurants on Long Island suffered a 50% drop in clientele. Prices for clams in unaffected areas were down 25. In New York's \$21.8 million fresh fish industry, clams are responsible for less than half the volume, but accounts for 80% of the total value. Approximately half of all the Mercenaria mercenaria in the country comes from New York waters which have not been touched by red tide toxicity. In order to abate the downward spiral in the seafood markets,

news releases were issued by the Shellfish Institute of North America (SINA). These were often misquoted, while lists of unaffected species, included with the bulletins were deleted from the published articles. To combat this, full page advertisements were placed in newspapers by individual seafood companies in order to alleviate public avoidance of safe species. SINA now has a system to keep the public informed and help lessen the aftershocks of future PSP outbreaks (Jensen, 1975).

Management

Outbreaks of PSP in North America have been documented since 1793 (Quayle, 1969). However, little was known of the source of the poison, and occurrences were sporadic. East coast Indians knew of PSP and associated the bioluminescence in water at night with outbreaks of PSP. Their management program required posting sentries at high ground, watching the ocean for glowing patches of water (Prakash, et al., 1971). This type of surveillance was not a reliable method. In 1799 100 Indians died within two hours of consuming mussels near Peril Bay, Alaska. It took six deaths and 102 cases during 1927 to stimulate definitive research regarding the PSP problem (Clem, 1975).

The majority of PSP outbreaks reported occur mainly on the east and west coasts of North America, the British Isles and Northern Europe (Wood, 1976). Of the North American outbreaks, those on the east coast tend to occur

with greater regularity than those on the west coast (Hunt, 1979). Those areas with erratic occurrences or extended periods of freedom from toxicity tend to curtail surveillance programs. This results in an increased health hazard when massive unexpected blooms occur (Clem, 1974). The cost of PSP analysis on a per sample basis was reported as approximately \$40.00 (Sherry, 1978). Multiplied by a number of stations and sampling several times a week, and the cost of management becomes prohibitive when there is seemingly no health risk.

Chile is facing this dilemma now. Following a bloom which caused the death of three fishermen, a monitoring program was initiated. Since then, there have been no PSP outbreaks, and the cost of the program is now considered prohibitive (Hunt, 1979). A similar situation existed in Washington state when several cases of PSP resulted from contaminated Vancouver clams. Washington now regularly monitors its own and Canadian shellfish.

In an evaluation of the public health risk, the Center for Disease Control in Atlanta reports PSP responsible for 1.1% of all known cases of food poisoning (Hughes, 1978). For the period 1971 to 1977, there were 12 outbreaks and 68 confirmed cases in the United States, despite monitoring programs. Most of the cases involved private collection. Only two of the outbreaks occurred with commercially distributed shellfish. In the United States, PSP monitoring is done in cooperation with state laboratories (Clem, 1979).

In Canada, however, all of the PSP analysis is conducted at the central laboratory in Ottawa. Receiving samples from the west coast sometimes requires a 7 to 10 day shipping time with further delays of three to five days to close the shellfish beds if toxicity is found (Hunt, 1979).

The level used for quarantine of shellfish is 80ug/100g shellfish meat. This level is accepted by the Food and Drug Administration (FDA), National Health and Welfare (NHW) of Canada and the World Health Organization (WHO) of the United Nations (Clem, 1974 ; Bond, 1958 ; Wood, 1976). However, this amount does not represent a clear and present danger in respect to public health. Rather, it indicates that a precipitous rise in the toxicity is likely (Hurst and Gilfillan, 1978). The LD-50 for humans has been estimated to be at levels of 0.5 to 1.0 mg. of pure toxin. A single mussel may contain this amount (Lutz, 1977; Prakash, et al. 1971; Shantz, 1975).

The fatality rate for PSP varies from estimates of 8.5% to 18% (Hughes and Merson, 1976; Wood, 1976). One factor which may contribute to the fairly low incidence of mortality is that shore residents in frequently affected areas show an acquired resistance which cannot be attributed to an immune response (Prakash, et al. 1971; Medcof, 1947)

Since it has been shown that quarantine levels are not hazardous to health, it has been suggested that those shellfish that have been harvested and found to contain toxicity between 80 and 100ug/100g. be allowed on the market

(Hurst, 1975). This would give enforcement authorities some opportunity for discretion in confiscation of shellfish. An 80 ug/100g. level would serve as an action level and prevent further harvest. The 100ug. limit would be the new maximum allowed for human consumption and the new quarantine level. This would allow for small errors inherent in the bioassay. However, there are cases where even the 80ug/100g. level is not low enough to provide an effective warning.

Mussels have an extraordinary ability to concentrate the toxin in a short time frame. *Mytilus* can increase from undetectable levels to over 500ug/100g. in 24 hours. *Mya* on the other hand can only increase from undetectable to 166ug/100g. in one day (Hurst and Gilfillan, 1978). Additionally, *Mya* does not reach as high a peak as *Mytilus* (Hurst and Gilfillan, 1978). In the three day interval between bioassays, it is clear that toxicity for mussels may become hazardous. This may not be the case for soft shells. Hurst (1978) reported that mussels from 5 of 12 stations sampling became toxic overnight. For the same bloom, soft shells showed only 1 in 7 stations turning toxic the day after first detection.

Information on species differences in toxicity has spawned the concept of species specific closures. There may be situations where mussels will be quite toxic while the soft shells may not be touched. *Spissula sclidissima*, the surf clam, has previously shown to retain toxicity for long periods. Extensive sampling would be required in a

management program for this species, but it would allow maximum utilization of resources.

The Maine Department of Natural Resources attempts to safeguard both the shellfishermen and the public health by utilizing over 119 sampling stations. These consist of 18 primary stations which always sample and function as low level indicators. Thirty five secondary stations are utilized if toxicity is detected at the primary stations. These are located in major clamming areas. The remaining tertiary stations are located in areas which give complete analytical coverage of the coastline (Hurst and Gilfillan, 1978). Partial closures can be implemented due to the large number of sampling sites. Reopening of the beds when toxicity declines to safe levels helps minimize economic loss (Lutz, 1977).

Other states do not maintain ambitious management programs. Alaska is one that has the potential for supporting a multimillion dollar clam industry that could be exploited on a year round basis. Much of Alaska's economy is seasonal in nature. In 1946, the discovery of PSP in canned butter clams collapsed the fledging industry that had started in 1930. Supporting an extensive PSP management program involves considerable cost. Understandably, the state is hesitant to fund this unless it can be certain that Alaskan employment opportunities can be expanded by exploitation. The razor clam is Alaska's only commercial food species. While there has been a long association of

PSP with west coast razor clams, the eviscerated shellfish have been canned and marketed without incident (Ritchie, 1977). Alaska has extensive mussel resources but they cannot be harvested due to the potential of PSP. A rapid field technique to determine the presence of the toxin is needed before economic development can be considered feasible (U.S. Dept. Comm., 1977; Prakash, et al. 1971).

In the state of Washington, the Quinault Indians maintain a limited razor clam industry which yields the tribe \$200,000 yearly. It is a self regulating management program that prohibits shellfishing between May and August, the period which is most likely to show toxicity (Ritchie, 1977).

In California , there is a coast-wide ban from May 1 to October 31 with bioassay monitoring on a monthly basis. The reason for the large interval is that California does not have a commercial shellfishing industry. Shellfishing there is a strictly recreational pursuit.

Environmental predictive index

While the FDA is supporting research involving an enzyme immunoassay system for the detection of PSP, a major thrust in PSP research is towards the development of an "environmental predictive index" (EPI) (Clem, 1979) . This would be an early warning system that could reduce the the number of mouse tests performed by a given agency. Yentsch and Glover (1978) reported some success in this direction by

associating blooms with the following : 1, existence of cells and cysts , 2, nitrogen and phosphorous 3, ferrous iron present in the seawater with 4, a characteristic absence of trace metals after 5, periods of heavy rainfall and resulting runoff. Since there is a direct correlation between the number of Gonyaulax cells and PSP, the EPI would be a tremendous forecasting aid.

Another approach that would take advantage of visual characteristics of the water during a bloom, involved the use of Nimbus G weather satellites . This method tried measuring ocean color and correlating this to chlorophyll distribution . This has not yet been successful at distinguishing Gonyaulax from other species. If the correct wavelength is found, PSP forecasting would be drastically simplified (Yentsch, 1979).

1. The purpose of this section is to provide a standard method for the analysis of PSP.

2. The method described herein is based on the use of a standard solution of PSP.

3. The standard solution is prepared by dissolving a known amount of PSP in a known volume of solvent.

4. The standard solution is used to compare the results of the analysis of the sample.

5. The results of the analysis are expressed in terms of the concentration of PSP in the sample.

APPENDIX II

**THE OFFICIAL METHOD FOR THE
ANALYSIS OF PSP**

1. The purpose of this section is to provide a standard method for the analysis of PSP.

2. The method described herein is based on the use of a standard solution of PSP.

3. The standard solution is prepared by dissolving a known amount of PSP in a known volume of solvent.

4. The standard solution is used to compare the results of the analysis of the sample.

5. The results of the analysis are expressed in terms of the concentration of PSP in the sample.

6. The method is applicable to the analysis of PSP in various samples.

7. The method is based on the use of a standard solution of PSP.

8. The standard solution is prepared by dissolving a known amount of PSP in a known volume of solvent.

9. The standard solution is used to compare the results of the analysis of the sample.

10. The results of the analysis are expressed in terms of the concentration of PSP in the sample.

Official method for the bioassay of paralytic shellfish poison. From "Recommended procedures for the examination of seawater and shellfish. 4th ed. Published by the American Public Health Association, 1970.

1.1 Materials

1.11 Paralytic shellfish poison standard solution (100 ug per ml): Request for PSP standard solution (100ug/ml) should be submitted to Division of Criteria and , Bureau of Water Hygiene, ECA, 12720 Twinbrook Parkway, Rockville, Maryland 20852.

1.12 Paralytic shellfish poison reference solution (11ug/ml): Dilute 1 ml standard solution to 100 ml with distilled water. Solution is stable several weeks at 3-4 degrees C. Final pH should be between 2.0 and 4.0.

1.13 Mice: Healthy mice, 19-21 g, from a stock colony are used for routine assays. If under 19 or over 21 g, apply correction factor to obtain true death time . Do not use mice weighing over 23 g. Do not reuse mice.

1.2 Standardization of bioassay

1.21 Dilute 10ml aliquots of 1 ug/ml reference solution with 10,15,20,25 and 30 ml distilled water, respectively, until intraperitoneal injection of 1 ml doses into a few test mice causes a median death time of 5-7 minutes; pH of dilution should be 2-4 and must not be over 4.5. Test additional dilutions in 1 ml increments of distilled water, e.g. if 10 ml diluted with 25 ml distilled water kills

mice in 5-7 minutes, test dilutions diluted 10+24 and 10+26. Care must be taken to maintain these dilutions between a pH of 2.0 to 4.0.

1.22 Inject group of 10 mice with each of 2, or preferably 3 dilutions that fall within death times of 5-7 min. Give one (1) ml dose to each mouse by intraperitoneal injection to the last gasping breath of mouse.

1.23 Repeat assay 1 or 2 days later, using dilutions prepared above which differed by 1 ml increments of distilled water. Then repeat entire test, starting with testing of dilutions prepared from newly prepared reference solutions.

1.24 Calculate median death time for each group of 10 mice used on each dilution. If all groups of 10 mice injected with any one dilution gave median death times of under 5 or over 7 min, disregard results from this dilution in subsequent calculations. On the other hand, if any of the groups of 10 mice injected with one dilution gave a median death time falling between 5 and 7 minutes include all groups of 10 mice used on that dilution, even though some of the median death times may be under 5 or over 7 minutes. From the median death time for each group of 10 mice, in each of the selected dilutions, determine the number of mouse units per 1 ml from table "6". Divide the calculated ug of poison per ml by the mouse units per ml to obtain conversion factor (CF) expressing ug poison equivalent to 1 mouse unit. Calculate the average of the

individual CF values and use this average value as a reference point to check routine assays. Individual CF values may vary significantly within a single laboratory if techniques and mice are not rigidly controlled. This situation will require continued use of reference standard, depending on the volume of assay work performed.

1.3 Use of standard with routine assay of shellfish

1.31 Check CF value periodically. If shellfish product are assayed less than once a week, determine CF values on each day assays are performed by injecting 5 mice with an appropriate dilution of the reference standard. If assays are made on several days during each week, then only one check need be made each week on a dilution of the standard such that the median death falls within 5-7 minute. The CF value thus determined should check with the average CF value within 20%. If it does not check within this range, complete group of 10 mice by adding 5 mice to the 5 mice already injected, and inject a second group of 10 mice with the same dilution of standard. Average the CF values determined for the second group with that of the first group. Take the resulting value as the new CF value. A variation of over 20% represents a significant change in the response on mice to the poison or in the technique of assay. Changes of this type require a change in the CF value.

1.32 Repeated checks of CF value ordinarily produce consistent results within 20%. If wider variations are found frequently, the possibility of untrcled or

unrecognized variables in the method should be investigated before proceeding with routine assays.

1.4 Preparation of sample

1.41 Clams, oysters and mussels: Thoroughly scrubbing the outside of shellfish with fresh water. Open by cutting adductor muscles. Rinse inside with fresh water to remove sand or other foreign matter. Remove meat from the shell by separating adductor muscles and tissue connecting at hinge. Do not use heat or anesthetics before opening shell, and do not cut or damage mollusk body at this stage. Collect ca. 100-150 g of meats in a glazed dish. As soon as possible, transfer meats to a No. 10 sieve without layering, and let drain for 5 minutes. Pick out the pieces of shell and discard drainings. Grinding household type grinder with one eighth to one quarter inch holes, or use blender until homogenous.

1.42 Scallops: Separate edible portion (adductor muscle) and apply test to this portion alone. Drain and grind as in 1.41.

1.43 Canned shellfish: Place entire contents of can (meat and liquid) in blender and blend until homogenous. For large cans, drain meat in large Buchner funnel or sieve and collect all liquid. Determine weight of meat and volume of liquid. Recombine portion of each in proportionate quantities. Blend recombined portions in blender until homogenous.

1.5 Extraction

1.51 Weigh 100g of well mixed material into a tared beaker. Add 100 ml 0.1N HCl, stir thoroughly and check pH (should be below 4.0 , preferably ca. 3.0). If necessary , adjust pH as indicated below. Heat the mixture , boil gently 5 min, and let cool to room temperature. Adjust cooled mixture to pH 2.0-4.0 (never above 4.5) as determined by BDH Universal Indicator, phencl blue, Congo red paper , or pH meter. To lower pH , add 5N HCl dropwise with stirring ; to raise pH, add 0.1N NaOH dropwise with constant stirring to prevent local alkalization and consequent destruction of the toxin. Transfer to graduated cylinder and dilute to 200 ml.

1.52 Return mixture to beaker, stir to homogeneity , and let settle until portion of supernatant is translucent and can be decanted free of solid particles large enough to block a 26 guage hypodermic needle. If necessary, centrifuge mixture or supernatant for 5 min at 3000 RPM or filter through paper. Only enough liquid to perform the bioassay is necessary.

1.6 Mouse test

1.61 Inoculate each test mouse intraperitoneally with 1 ml of the acid extract . Note the time of inoculation and observe the mice carefully for time of death as indicated by the last gasping breath. Record death time from stopwatch or clock with a sweep second hand. One mouse may be used for the initial determination, but 2 or 3 are preferred. It the death time , or the median death time of several mice is

under 5 min, make a dilution to obtain death times of 5-7 minutes. If the death time of 1 or 2 mice injected with undiluted sample is over 7 minutes, a total of three or more mice for each injection must be inoculated to establish the toxicity of the sample. If large dilutions are necessary, adjust the pH of the dilution by the dropwise addition of dilute HCl (0.1 or 0.101N) to pH 2.0-4.0, never above 4.5. Inoculate 3 mice with a dilution that gives death times of 5-7 minutes.

Calculation of toxicity

1.71 Determine median death times of mice, including survivors, and determine corresponding number of mouse units. If test animals weigh below 19g or above 21g, make correction for each mouse by multiplying mouse units corresponding to death time for that mouse by the weight correction factor for that mouse and then determine the median mouse unit for the group. (consider the death time of survivors as over 60 min or equivalent to less than 0.875 mouse units in calculating the median.) Convert mouse units to ug poison per ml by multiplying by the CF value.

1.72 $\text{ug poison per 100g meat} = (\text{ug per ml} \times \text{dilution factor}) \times 200.$

1.73 Consider any value over 80 ug per 100 g as hazardous and unsafe for human consumption.

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APPENDIX III

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