CONTRIBUTED PAPERS

Chemical Indices of Decomposition in Flounder

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Previous communications from this laboratory have described and evaluated the progressive deteriorative changes that occur in cod (1), haddock (2), and ocean perch (3) as part of the work that has been in progress in the Food and Drug Administration for several years on the development of chemical methods for detecting decomposition in fishery products. This paper extends the work to flounder, and uses the previously described procedures for the preparation of authentic packs and methods of analysis for volatile amines and volatile bases.

Methods of Analysis

Experience has shown that volatile acids higher in molecular weight than acetic acid are not found in pelagic fish processed on the east coast. This observation is utilized as the basis for simplifying methods of analysis for this type of fish by eliminating the chromatographic separation step designed to separate and determine individually the volatile fatty acids other than formic. After proper corrections are made in the titration of the distillate for the formic acid present, acetic acid can be computed directly from the corrected titration (4–7). Previous work has also shown that succinic acid and alcohol in east coast pelagic fish show less correlation with organoleptic judgment than the other indices discussed in this and previous papers. For this reason, these two determinations have not been applied to the two flounder packs reported here.

ALTERNATIVE METHOD FOR ACETIC ACID IN PELAGIC FISH

Apparatus

See **18.015.**[1]

Reagents

See **18.016.**

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[1] References are the section numbers of *Official Methods of Analysis*, 9th Ed., Association of Official Agricultural Chemists, 1960.

Standardization of Distillation Apparatus and Preparation of Solution

See **18.017**; **18.018**.

Distillation and Computation of Volatile Acid Number

Proceed as in **18.019** except collect two 100 ml portions of distillate and titrate with 0.01*N* $Ba(OH)_2$.

Determination of Individual Fatty Acids

Combine the two 100 ml portions of titrated distillate and proceed as in **18.020** except to calculate formic acid originally present in aliquot of sample in distillation flask before distillation, by dividing mg of formic acid found by 0.405 (fraction formic acid distilled in 200 ml distillate), and multiply by 4 to obtain formic acid in 100 g sample being analyzed.

Computation of Acetic Acid

(1) Formic acid present.—To obtain ml 0.01*N* formic acid in 200 ml of distillate, divide weight of formic acid obtained in 200 ml distillate by 0.46. Subtract the result from the combined titrations of the two 100 ml portions of distillate (corrected for their blank). Divide this quantity by 0.57 (fraction of acetic acid in 200 ml distillate) to obtain ml of 0.01*N* acetic acid in distillation flask before distillation. To obtain mg of acetic acid multiply by 0.0006, and to obtain mg of acetic acid per 100 g multiply the result by 4.

(2) Formic acid absent.—Proceed as in *(1)* omitting correction for formic acid.

Results

Table 1 presents the data obtained by analysis of flounder held under ice, packed

Table 1. Studies of progressive decomposition in flounder

Fish No.	Age in Days	Organoleptic Class	Fish Temp., °F	Air Temp., °F	Volatile Acid No. "VAN"	Volatile Acids		Volatile Bases, ml 0.01*N*/ 100 g	Volatile Amines, ml 0.05*N* $KMnO_4$ per 100 g	Trimethylamine (TMA), mg *N*/ 100 g
						Formic, mg/ 100 g	Acetic mg/ 100 g			
					Pack No. 1					
1	0	0	47	70	7	0.0	7.9	71	103	4.2
2					4	0.0	4.5	70	87	3.1
3					4	0.0	4.0	60	49	3.3
4					4	0.0	4.5	56	48	2.4
5					6	0.0	6.8	75	53	3.9
1	1	0	43	69	6	0.0	6.8	77	82	3.2
2					7	0.0	7.2	72	43	1.9
3					7	0.0	7.9	60	38	3.3
1	2	1	33	73	7	0.0	6.9	76	—	2.4
2					12	0.0	12.2	77	22	2.4
1	3	1	43	74	10	0.0	10.3	31	54	3.7
2					11	0.0	11.2	54	56	3.3
3					12	0.0	12.8	45	51	4.6
4					10	0.0	10.1	89	64	4.7
5					10	0.0	10.2	42	64	3.2
1	4	1	33	74	9	0.0	9.9	48	65	3.1
2					8	0.0	8.6	50	48	2.3
1*	5	1	33	74	22	0.0	23.1	139	242	19.3
2					26	0.0	27.3	77	162	16.8
1	6	1	33	69	21	0.0	21.8	91	159	11.4
2					26	0.0	26.9	89	201	14.0

Table 1. (*Continued*)

Fish No.	Age in Days	Organ-olep-tic Class	Fish Temp., °F	Air Temp., °F	Volatile Acid No. "VAN"	Volatile Acids Formic, mg/100 g	Acetic mg/100 g	Volatile Bases, mg 0.01*N*/100 g	Volatile Amines, ml 0.05*N* $KMnO_4$ per 100 g	Tri-methyl-amine (TMA), mg *N*/100 g
					Pack No. 1					
1	7	1	33	71	24	0.0	25.4	104	91	15.6
2					26	0.0	27.8	131	82	23.9
1	8	1	33	73	18	trace	19.2	107	210	12.8
2					17	trace	17.4	65	126	7.1
3					11	trace	12.1	54	80	6.0
1*	9	1	33	74	35	3.5	34.1	87	395	28.2
2*					49	8.4	42.7	124	250	34.2
1*	10	1	33	71	40	5.2	34.0	124	258	24.5
2*					35	3.4	33.8	110	266	28.1
1	10	2	33	71	56	7.4	52.3	112	258	34.4
1*	11	1	33	71	34	3.2	32.4	103	244	26.1
1	11	2	33	71	35	4.1	32.8	122	248	43.8
2					53	5.9	50.1	107	229	36.5
3					59	7.5	55.0	137	292	40.6
1	12	2	33	71	46	4.0	44.8	140	408	35.4
2					68	3.8	68.5	128	403	33.1
3					57	13.4	45.8	96	247	41.4
4					40	3.2	39.0	143	326	29.9
5					32	3.7	30.4	134	356	22.7
1	12	3	33	71	76	9.5	62.4	197	296	43.6
1	13	2	33	66	55	7.4	50.8	157	257	35.7
2					39	3.6	37.6	122	324	30.3
1	14	2	33	66	35	5.5	32.1	161	315	32.3
1	15	2	33	68	25	trace	26.0	144	408	28.8
1	16	2	46	68	26	4.5	22.7	225	334	29.0
1	17	3	63	68	29	9.4	21.3	165	274	17.0
2					23	4.5	19.8	161	260	22.0
3					28	4.7	25.1	195	321	30.7
4					32	4.5	29.0	189	365	31.7
5					25	4.9	21.6	155	277	19.8
					Pack No. 2					
1	0	0	50	74	6	0.0	6.7	49	22	1.0
2					7	0.0	7.1	62	37	0.8
3					8	0.0	8.0	53	32	1.6
4					7	0.0	7.0	51	24	1.1
5					7	0.0	7.3	65	42	1.2

(Continued)

Table 1. *(Continued)*

Fish No.	Age in Days	Organoleptic Class	Fish Temp., °F	Air Temp., °F	Volatile Acid No. "VAN"	Volatile Acids		Volatile Bases, mg 0.01*N*/100 g	Volatile Amines, ml 0.05*N* $KMnO_4$ per 100 g	Trimethylamine (TMA), mg *N*/100 g
						Formic, mg/100 g	Acetic mg/100 g			
Pack No. 2										
1	1	0	34	73	6	0.0	6.3	53	37	1.7
2					7	0.0	7.7	43	43	1.3
1	2	0	33	74	6	0.0	6.7	65	63	1.1
2					6	0.0	6.6	91	25	1.5
1	4	0	33	71	10	0.0	10.0	54	44	6.1
2					11	0.0	11.5	92	56	3.2
1	5	0	33	73	8	0.0	8.0	64	39	1.7
2					7	0.0	7.5	61	42	2.0
1	6	0	33	74	12	0.0	12.7	70	55	6.2
2					15	0.0	15.3	87	113	10.6
1	6	1	33	74	25	trace	26.2	116	63	32.5
1	7	1	33	71	11	trace	11.6	66	58	3.4
2					11	0.0	11.4	43	51	4.6
3					17	trace	18.4	49	92	8.1
4					21	trace	22.0	56	116	18.6
5					15	trace	16.0	73	101	13.7
1	8	1	33	71	26	trace	27.6	135	244	21.4
2					24	trace	25.2	122	234	10.9
1	8	2	33	71	47	6.3	43.7	134	338	34.5
1	9	1	33	71	13	trace	13.4	124	262	17.7
2					11	0.0	11.8	88	207	12.8
1	10	1	33	66	12	0.0	12.3	103	190	20.3
2					16	0.0	17.0	102	194	20.9
1	10	2	33	66	30	3.2	32.7	161	435	27.3
1	11	2	33	66	36	6.7	31.6	173	362	34.2
2					29	4.8	25.6	188	392	44.4
3					73	12.0	65.3	170	351	30.1
4					66	13.0	57.7	173	343	41.9
5					56	11.1	48.4	206	389	41.9
1	11	3	33	66	85	16.9	73.8	316	545	47.0
2					79	18.8	65.3	218	353	42.7
3					86	19.8	72.2	202	456	40.3
4					63	12.2	54.6	268	335	31.4
5					75	15.7	64.1	201	427	56.4

* Samples marked with asterisks represent fish which, on later examination, were found to have gone from the Class 1 designation recorded for them at the time of filleting to Class 2 as a result of delay between filleting and freezing.

in 1959. In the first pack the fish reached Class 1 in three days, while Class 2 was not reached until the eleventh and twelfth days. A considerable number of fillets classed as 1 at time of filleting were found to have gone to Class 2 when they were prepared for chemical examination; this was due to delay between filleting and freezing. These samples

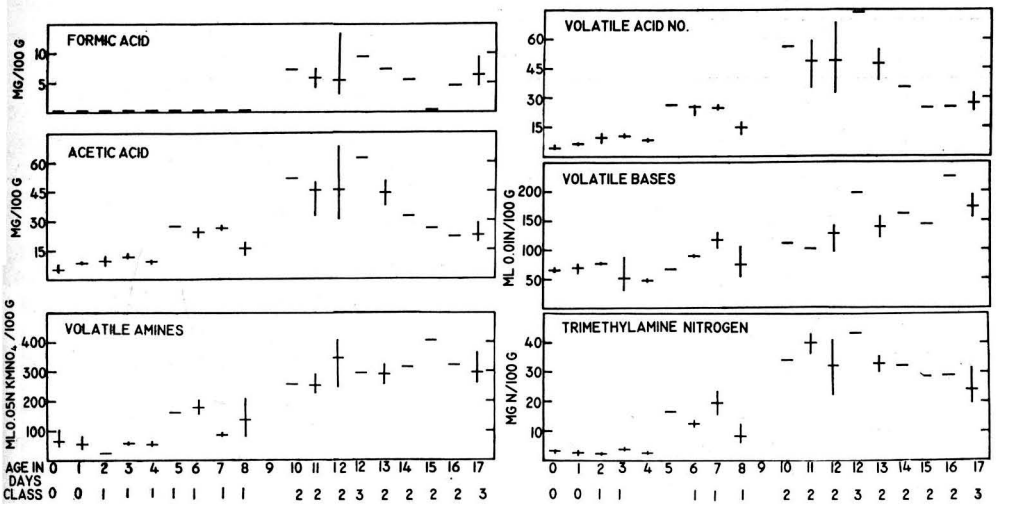

Fig. 1—Day-to-day study of progressive decomposition in heavily iced flounder.

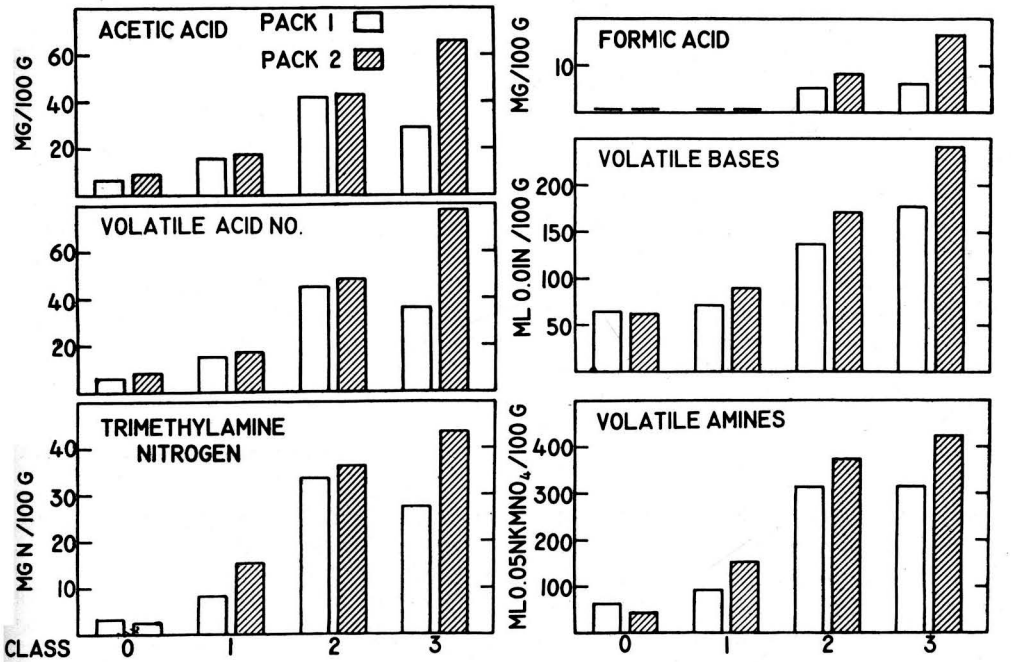

Fig. 2—Average values for indices of decomposition in flounder according to organoleptic classification and type of handling.

are marked with an asterisk in the table. In the second pack the fish reached Class 1 on the seventh day, and reached Class 2 on the tenth and eleventh days. While there were increases in some indices when the fish were in Class 1, particularly with the second pack, these increases were not in the range found for the indices for Class 2 (decomposed).

Formic acid was not found until the fish had reached Class 2 (decomposed).

The data obtained on the day-by-day study of progressive decomposition in the first pack are presented graphically in Fig. 1. The ranges for each index determined, showing the organoleptic class and the days of holding, are represented by the vertical bars; the average is indicated by the horizontal bars.

The averages of each of the indices for each organoleptic classification are presented graphically in Fig. 2.

Summary

A study of the individual analyses of progressive decomposition packs of flounder leads to the conclusion that volatile acid number (VAN), formic and acetic acids, volatile bases, volatile amines, and trimethylamine (TMA) show a high degree of correlation with organoleptic judgment.

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The Nutritive Value of Fish Meal Protein: A Comparison of Chemical Measurements with a Chick-Feeding Test

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Numerous investigations conducted in recent years have attempted to measure the nutritive value of protein in fish meals. As yet, no chemical or biological method for determining the nutritive value is fully accepted. Biological methods, despite the length of time required to conduct them, are usually employed because they are considered more reliable. They ordinarily involve including the protein meal either as a sole source of protein or to provide a part of the protein in poultry feed and then measuring the growth of young chicks fed the feed under controlled conditions. A chemical method for measurement of the quality of protein feedstuffs has recently been adopted as first action by the Association of Official Agricultural Chemists under the title of "Determination of the Pepsin Digestibility of Animal Protein Feedstuffs" (4), and is being used by some laboratories for control of protein quality. Estimates of the nutritive value of protein meals have also been based on the protein, ash, or crude fiber content of the meals.

The purpose of this paper is to compare results obtained from studying a number of fish meals using (a) the pepsin digestibility test, (b) a chick-feeding test, and (c) other chemical analyses.

EXPERIMENTAL

Fish meal samples used.—In 1958, workers at the Seattle Technological Laboratory of the Bureau of Commercial Fisheries used a laboratory pilot plant to prepare a series of meals from cooked herring press cake and cooked tuna waste press cake, two of the principal sources of fish meal on the Pacific Coast. The press cakes were converted to meals by exposure to direct heat in a rotating drum at tem-

peratures ranging from 185° to 395°F for periods ranging from 20 to 180 minutes (Table 1). The different processing conditions subjected the meals to varied degrees of heat treatment and produced meals of widely differing moisture content and nutritive value—much greater than variations found in commercial meals. The prepared meals were sealed in plastic bags, cans, or bottles and kept under refrigeration until used in the tests.

Methods of analysis.—Proximate analyses were performed by the State Feed Control Laboratory of the California Department of Agriculture by official methods of the Association of Official Agricultural Chemists (1).

Pepsin digestibility test.—The procedure developed by Gehrt and associates (2) for determining the pepsin digestibility of protein in meals had just been adopted as first action by the Association of Official Agricultural Chemists (4) at the time the feeding tests were completed. This first action procedure was used in this study. Briefly, it involves removing the fat from a 1 g sample of the meal with successive portions of ethyl ether; treating the defatted sample with 150 ml of a solution containing 0.2% pepsin in 0.075*N* HCl at 45°C for a period of 16 hours under constant agitation; filtering the residue on a tared paper; and then drying, weighing, and analyzing the residue for crude protein by the Kjeldahl procedure. The per cent of protein digested by the pepsin was calculated as the difference between the amount of protein in the meal and the amount of protein in the residue, divided by the amount of protein in the meal, and multiplied by 100.

Chick-feeding test.—The meals were used in a chick-feeding test conducted at the Department of Poultry Husbandry, University of California, Davis. The fish meals were included to provide 20% protein as the sole source of protein in a diet composed of purified ingredients (3). The diets were fed for 8 days to 11 day old uniformly sized White Leghorn chicks kept in cages at a room temperature of about 85°F, with 12 hours of continuous artificial light provided each day. Three groups of 4 chicks each (3 cages) were fed each diet. The rate of growth of the chicks was expressed as:

$$\frac{(\text{final weight} - \text{initial weight}) \times 100}{(\text{initial weight} + \text{final weight}) \div 2 \times \text{days}} = \text{per cent gain per day.}$$

Results and Discussion

Results obtained from the pepsin digestibility test and the growth of the chicks fed diets containing the meals are presented in Table 1, together with the drying conditions used to prepare the meals, and the moisture, protein, ash, and crude fiber[1] content of the meals.

All the protein in the cooked herring press cake was pepsin digestible. Due to the high moisture content of the press cake, however, it was not transported to Davis for feeding.

The pepsin digestibilities for the six herring meals dried at temperatures of 230°F or lower were almost identical. The herring meal dried at 275°F showed a lower pepsin digestibility. Growth of the chicks fed the meals showed no relationship to the pepsin digestibility or to the drying conditions used in preparing the meals. To be statistically significant, differences in chick growth of approximately 1% gain per day are required. Neither the protein, ash, nor crude fiber content of the meals showed any consistent relationship to pepsin digestibility, chick growth, or drying conditions.

All the tuna meals showed a consistent decrease in both pepsin digestibility and the chick growth they allowed with each increase in the drying temperature used to prepare the meals. The decrease in pepsin digestibility, however, was relatively much smaller than the effect on chick growth for the first three meals. Differences in the drying conditions used to prepare the tuna meals were much greater than those used for the herring meals. The drastic drying conditions used to prepare the last tuna meal resulted in low pepsin digestibility, high crude fiber content, and no chick growth.[2] This meal was very definitely scorched.

[1] Fish meals contain no fiber unless they are adulterated with fiber-containing matter. The method used to determine crude fiber will show small amounts of acid- and base-insoluble matter normally present in the meals. However, it has been noticed that charred material is often left as a residue and an indication of the amount of char may be given by the crude fiber determination.

[2] The fact that this meal allowed no chick growth does not mean that its nutritive value is zero. Since the chicks were able to maintain their weight, it had a small nutritive value. Chicks kept under the conditions of this experiment and fed diets containing no protein lose about 3% of their weight each day.

Table 1. The protein evaluation and chemical composition of fish meals produced under varied conditions

Sample Number	Drying Conditions: Final Temperature (°F)	Drying Conditions: Time to Reach Final Temperature (Min)	Drying Conditions: Time Held at Final Temperature (Min)	Composition: Moisture %	Composition: Protein (% of Dry Matter)	Composition: Ash (% of Dry Matter)	Composition: Crude Fiber (% of Dry Matter)	Protein Evaluation: Pepsin Digestibility (% Digested)	Protein Evaluation: Chick Growth (% Gain/Day)
Herring									
1	(Presscake, not dried)		—	65.4	84.3	13.5	0.3	100.0	[a]
5	230	50	0	15.2	74.5	12.6	0.6	97.3	5.9
6	230	42	20	5.2	79.5	11.9	0.5	97.0	6.6
3	215	35	0	15.6	73.7	8.8	0.7	96.8	6.0
4	215	45	20	5.4	79.5	9.5	0.8	96.5	5.3
2	185	21	0	42.0	82.5	9.5	1.4	96.1	6.1
7	275	55	0	4.9	80.2	11.6	1.1	92.7	6.2
Tuna									
8	185	20	0	2.4	57.4	19.2	0.6	94.6	5.9
9	215	25	0	8.8	65.9	22.3	0.7	93.9	4.9
10	245	35	0	3.3	62.6	21.4	1.1	91.7	3.6
11	395	[b]	180	2.2	58.9	24.8	23.6	24.9	0.0

[a] Not determined.
[b] This meal was deliberately scorched. Drier was set at maximum temperature and presscake dried for 180 minutes. Length of time required by drier to reach 395° was not determined.

Summary

A study was made of the possible relationships between the results obtained from (a) the first action AOAC method for determining pepsin digestibility of animal protein feedstuffs, (b) the growth of chicks fed the meals, and (c) other chemical analyses, using six herring and four tuna meals prepared in the laboratory under a wide variety of carefully controlled conditions.

The six herring meals averaged 6% gain in weight per day per chick and 96% digestible protein. Deviations from these averages were moderate for growth effect and slight for digestibility. When a meal was produced under extreme processing conditions, as was the case with one tuna meal, both pepsin digestibility and the chick growth possible with the meal were drastically lowered.

Crude fiber content of a fish meal higher than one per cent may be caused by overheating during processing and may indicate a lowered nutritive value. There was apparently no consistent relationship between the protein or ash content of the meals tested and their nutritive value as measured either by pepsin digestibility or chick growth.

Acknowledgments

The authors sincerely appreciate the assistance rendered by Van P. Entwistle, Beverly D. Lundholm, Neva L. Karrick, C. R. Grau, and Mabel A. Edwards in this study.

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Species Identification by Starch Gel Zone Electrophoresis of Protein Extracts. I. Fish*

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The use of zone electrophoresis for the separation of complex protein fractions is becoming more and more prevalent. The advantages of zone over moving boundary electrophoresis have been aptly described by Smithies (1) in his development of the use of starch gel as a supporting medium. This method gives a higher resolution of proteins and more consistency in pattern development.

The practical application of starch gel electrophoresis has been reported by several workers in the enzyme field (2–4). Both esterases and phosphatases have been separated from blood serum and homogenized tissue. Histochemical methods are used to indicate the presence and position of the enzyme bands resolved in the starch strips.

There has existed a need for a direct and simple method for the identification of specific proteins such as those from fish, meat, etc. The species specificity of fish has been demonstrated with some success through the use of moving boundary electrophoresis by Connell (6) and later by Dingle (7). These workers used a water extract of protein obtained from the fillets. Moving boundary electrophoresis has also given characteristic patterns on plasma proteins of amphibia and reptilia (8). The use of paper partition chromatography in taxonomic studies of fresh tissue of several species of fish was made as early as 1953 (9).

Recently we have reported (5) on the identification of fish species by using starch gel zone electrophoresis. We have been able to identify various species of food fish with certainty. Extracts from a particular species give characteristic bands and the pattern obtained from perch extracts differs greatly from that of haddock or whiting. Of the available species of frozen food fish thus far examined by this method—perch, flounder, cod, haddock, whiting, pollock and halibut—we have yet to find two whose patterns are similar with respect to band spacing or number of bands (Fig. 1).

Information obtained from these findings would be of great value in determining the authenticity of marketed frozen fillets and would help in preventing the substitution of a cheaper species for the more costly ones. A case of substitution was detected in Kansas where a merchant advertised catfish while actually selling pollock (Fig. 2).

This method is also effective when applied to a mixture of species (as may be used in fish stick stock). The pattern obtained in these cases shows more bands than an individual species will show.

METHOD

Sections of fish taken from frozen fillets of authentic samples were blended with twice their weight of distilled water for several minutes, i.e., 20 grams plus 40 ml. The mixture was then centrifuged at 1500 rpm for five minutes. The clear supernatant was removed and used as the protein extract for electrophoretic analysis.

Starch gel trays were prepared from a 12% soluble starch according to the method of Smithies (1) in a 0.03*M* borate buffer of pH 8.6.

Strips of filter paper 6 × 19 mm (S&S #598) saturated with the fish protein extract were inserted crosswise in the starch gel midway in the trays. The trays were placed in an ice water cooled electrophoresis cabinet (10°C) and subjected to a current of 15 ma–200 v. for a period of 5 hours. The starch strips were then removed and cut for staining.

The cut starch strips were immersed in a saturated solution of Amido Black 10B in methanol-water-acetic acid solvent (5:5:1) for 5 minutes. The dye solution was then poured off, and the strips washed for four to five hours with the solvent mixture, either by continuous

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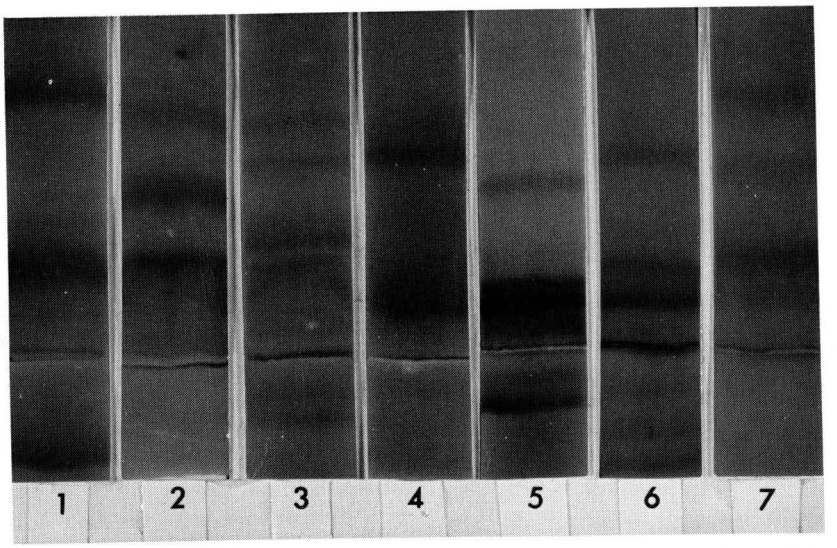

Fig. 1—Band patterns of various species of fish. (1) Perch; (2) Cod; (3) Haddock; (4) Whiting; (5) Pollock; (6) Halibut; (7) Flounder.

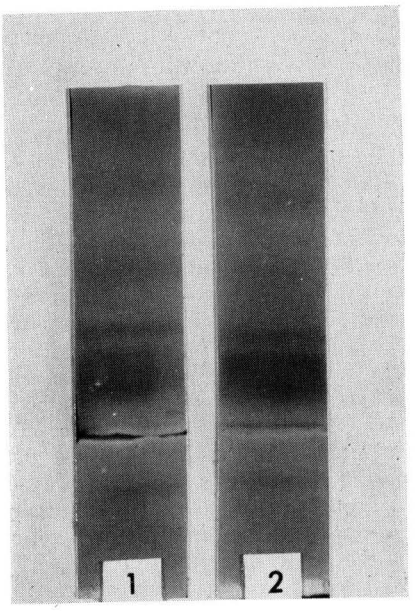

Fig. 2—Identification of pollock sold as catfish. (1) Pollock (authentic sample); (2) Sample.

circulation (10) or by fresh half-hourly changes. The solvent was then washed from the strips with running tap water for an hour effecting further clearing.

The finished patterns can then be photographed (or schematic drawings made) so as to give a permanent record. Photos of unknown species may then be compared with those of known species and identification made. Under uniform conditions, such as current, time, starch concentration, and extract strength, our results have been reproducible. Cooked or processed fish, as expected, give negative results, due to the denaturing of the protein.

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Fluorometric Determination of Added Pyridoxine in Enriched White Flour and in Bread Baked From It

By DOUGLAS J. HENNESSY, ANNE M. STEINBERG, GEORGE S. WILSON, and WILLIAM P. KEAVENEY (Department of Chemistry, Fordham University, Bronx 58, N.Y.)

The fluorometric determination of pyridoxine described by Fujita, *et al.* (1) has been simplified in certain respects and improved in others so that it now applies to the routine analysis of white flour enriched by the addition of pyridoxine, thiamine, riboflavin, niacin, and iron as well as bread made from this flour. Only pyridoxine responds to the analytical procedure which is described.

The simplification lies (*a*) in the design of the exchange columns so that a capillary is used in place of a stop-cock to control flow rate; (*b*) in the extraction of the sample where autoclaving with 0.6*N* H_2SO_4, neutralization of the acid with $Ba(OH)_2$, and centrifuging are omitted; and (*c*) in the acid elution of the pyridoxine from the Decalso column with cold rather than boiling elutriant.

The improvements are (*a*) in the heating of the blank to eliminate effervescence which interferes with fluorometry and (*b*) in the prevention of precipitation of the hydrated oxides of manganese and aluminum when the pH is brought to approximately 9.5. This is accomplished by the use of Versene[1] and Rochelle salt and eliminates the need for centrifugation in the entire analysis.

Pyridoxine determinations by this modified procedure were conducted on samples of white flour and bread baked from it. Known amounts of pyridoxine HCl were added to some samples while other samples had no pyridoxine added. The results of these analyses are tabulated with the results of the microbiological assay of the same samples. The microbiological assay uses a more vigorous extraction method and measures vitamin B_6 in all its forms, which accounts for the significantly greater amounts of vitamin B_6 found by this method. The two methods agree relatively well in estimating added pyridoxine with regard both to replicate results and to the actual supplement. (*see* Table 1). Expressed as standard error, the statistical uncertainty of the microbiological assay is generally not better than 10%. The chemical method has a standard error nearer to 3%.

[1] We are grateful to Robert G. Kelly for suggesting Versene as a complexing agent suitable for this purpose.

METHOD

Reagents

(a) *H_2SO_4, 3.6N.*—Dilute 100 ml concd H_2SO_4 to 1 L with distilled water.

(b) *$NaC_2H_3O_2 \cdot 3H_2O$, 4M.*—Dilute 544 g reagent grade salt to 1 L with distilled water.

(c) *Mylase P.*—(Wallerstein Co., Staten Island 3, N.Y.) 6% solution in distilled water made up fresh as needed.

(d) *H_2SO_4, 0.22N.*—Dilute 6 ml concd H_2SO_4 to 1 L with distilled water.

(e) *Isopropyl alcohol.*—Distill so that the distillate does not come in contact with stopcock grease or rubber.

(f) *$KMnO_4$, 2%.*—Dissolve 20 g reagent grade salt in distilled water and dilute to 1 L.

(g) *H_2O_2, 3%.*—Dilute 30% hydrogen peroxide 1:10 in small amounts as needed.

(h) *HCl, 9N.*—Make 750 ml concd HCl to 1 L with distilled water.

(i) *Quinine reference working standard.*—Dilute quinine in 0.1*N* H_2SO_4 to make 10 mmg/100 ml solution.

(j) *Complexing reagent.*—Dissolve 200 g sodium salt of ethylenediamine tetraacetic acid (Versene) and 200 g potassium sodium tartrate (Rochelle salt) in distilled water and dilute to 1 L. Stir solution 20 minutes with each of four 20 g portions of decolorizing charcoal (Norit-A), and filter by suction between each charcoal treatment.

(k) *NH_3 water, 8N.*—Dilute 500 ml concd ammonia water (28%) to 1 L with distilled water.

(l) *Activated Decalso.*—Wash 200 g of 60–80 mesh Decalso (Permutit Co., New York, N.Y.)

by decantation three times with three 1 L portions of water, pouring off the extreme fines with the supernatant wash water. Pour 3 L boiling 0.2*N* HCl on the Decalso and, while stirring mechanically, keep mixture at the boil for 10 minutes. Discard supernatant liquid and repeat hot acid treatment of Decalso in same manner until Decalso has received six hot acid treatments, each time for ten minutes. The 3rd, 4th, and 5th acid treatments extract the iron from the Decalso, and in all extractions except the last considerable aluminum ion is removed from the Decalso. After washing with three 2 L portions of distilled water, the Decalso is ready for use and may be stored wet.

Any solution which makes an unusually high fluorescent contribution to the final reading must be made from a different sample of reagent or it must be treated with decolorizing charcoal to remove fluorescence.

Apparatus

(a) *Fluorometer, galvanometer, transformer, voltage regulator, filters as for thiamine determination, three cuvettes.*[2]

(b) *Waring-type blender.*

(c) *Ion exchange tubes.*—Fifteen with 15 × 0.7 cm barrel, 50 ml reservoir at upper end and 3 × 0.04 cm capillary at lower end; stand to hold tubes.

(d) *Test tubes.*—Two dozen Pyrex, 2 × 20 cm graduated at 22 ml mark.

(e) *Fountain to flush used Decalso from columns.*—12″ length of 5 mm (o.d.) tubing connected to water outlet. Remove gauze from upper end of Decalso bed. Invert exchange tube onto fountain to wash out Decalso.

Preparation of Flour Extracts

Mix 5 g sample of flour with 25 ml distilled water to give a smooth paste. Add an additional 50 ml distilled water and 1 ml of 3.6*N* H_2SO_4, and heat mixture for 30 minutes in a boiling water bath with frequent mixing during the first 15 minutes. Cool mixture to about 50°C and add 2 ml 4*M* sodium acetate.

Preparation of Bread Extracts

(a) *Fresh bread.*—Take a 50 g sample of bread so as to obtain a representative proportion of crust and crumb. Take sample quickly so that bread does not significantly change weight by loss of moisture during the sampling. Add sample to 300 g water in a Waring Blendor and blend to a smooth paste. Transfer 50 g of this paste to a 100 ml volumetric flask with about 25 ml rinse water. Add 1 ml 3.6*N* H_2SO_4 and heat mixture in boiling water bath for 30 minutes. Cool to 50°C and add 2 ml 4*N* sodium acetate.

(b) *Dried bread.*—Pulverize dry bread and then treat as for preparation of flour extracts.

Digestion

(a) Add 5 ml freshly prepared 6% solution Mylase P to the extraction mixtures, prepared as described above. Incubate resulting mixture at 50°C overnight (or 4 hours minimum).

(b) Cool to room temperature and dilute to 100 ml with distilled water, filter digested mixtures, discard the first 15 ml of filtrate, and collect the next 40–50 ml.

Ion Exchange Purification

Place a small plug of folded 1 × 1″ gauze in the bottom of the exchange tube, using a glass rod. Add distilled water to the tube until the reservoir is about half filled. Introduce wet activated Decalso so that the 7 mm column is filled to within about 1 cm of the top with settled Decalso. Put a second plug of gauze onto the upper surface of the Decalso. Pour water out of the reservoir and lay exchange tube on its side until ready for use. (If left upright, Decalso will drain, and undesirable air pockets will form between the Decalso grains in the upper part of the column.)

Introduce a 10 ml, 15 ml, or 20 ml aliquot[3] of flour or bread extract into the reservoir of an upright exchange tube and allow to flow through the Decalso by gravity. Wash column with two separate 10 ml portions of distilled water, and allow them to flow through. Place a 10 ml portion of 0.22*N* H_2SO_4 in the reservoir and collect effluent in a 25 ml volumetric flask. When the first 10 ml acid has drained through so as to reach the upper surface of the Decalso, put a second 10 ml portion of 0.22*N* H_2SO_4 into the reservoir. When this has drained through the Decalso, use a little less than 5 ml acid to finish the elution. Dilute effluent to 25 ml with 0.22*N* acid and mix well.

Oxidation

Pipet two 10 ml aliquots of the acid effluent from the ion exchange purification into each

[2] Pfaltz & Bauer, Inc., New York, N.Y. fluorophotometer and accessories were used in this investigation.

[3] The 10 ml, 15 ml, or 20 ml aliquots, respectively, are recommended when the pyridoxine HCl has been added to the flour or bread at levels of approximately 3.0 mg, 1.5 mg, or 0.0 mg per pound.

of two tubes graduated at 22 ml. Add 1 ml isopropyl alcohol to each tube and cool mixture in an ice-water bath to 0–2°C. Add a 2 ml portion of ice cold 2% potassium permanganate to each tube from a quick delivery pipet and mix. After 50 seconds, add, with mixing, 0.5 ml of 3% H_2O_2 to discharge excess permanganate. Reserve one tube and its contents for lactonization. Put the other tube (the blank) into a boiling water bath for 2 minutes to drive off oxygen so that effervescence will not interfere with the fluorescent reading. Cool blank in ice-water to 0–2°C, add 2 ml of 9*N* HCl and 2 ml complexing reagent with mixing, and add enough 8*N* NH_3 water to make volume to 22 ml. Place 15 ml of this mixture in the cuvette of the fluorometer for determining blank fluorescence (d_0). Use second tube for lactonization step.

Lactonization

Add 2 ml 9*N* HCl to the second tube (*see* Oxidation) and place mixture in a boiling water bath for 12 minutes. Then cool tube in ice-water to 0–2°. Add 2 ml complexing reagent and then enough 8*N* NH_3 water to make the volume to 22 ml. Use a 15 ml portion of the mixed solution for fluorometry.

Fluorometry

As the reference working standard, employ a solution of quinine in 0.1*N* H_2SO_4 (0.1 mmg/ml), or a standard fluorescent glass block such as is used for a standard in the thiamine determination. Prior to taking galvanometer deflections on samples, set standard at some convenient deflection with the iris diaphragm open, for example, to position 30.

d = galvanometer deflection of sample

d_0 = galvanometer deflection of blank

d_s = galvanometer deflection of reference working standard

k = proportionality constant

$k \frac{d-d_0}{d_s}$ = mmg pyridoxine HCl in 10 ml aliquot

Determination

To evaluate k, use crystalline pyridoxine HCl as a primary standard. Dilute a stock standard (10 mg pyridoxine HCl/100 ml) in 0.2*N* H_2SO_4 with a diluent suitable to the step at which the pyridoxine is introduced in the analytical procedure.

Pure pyridoxine HCl, either dissolved in 0.2*N* H_2SO_4 or added to the acid eluate of the ion exchange from determination of a non-enriched flour or bread at levels of 0.5–5.0 mmg/10 ml aliquot, gave the same values for k, *viz.*, 2.8 ± 0.1.

Addition of pyridoxine HCl to the extract of non-enriched flour or bread at any step prior to the ion exchange gave k values of 3.0 ± 0.2 and 3.1 ±0.2, respectively. Thus the recovery of pyridoxine at the ion exchange step is 93 ± 8% for flour assay and 89 ± 8% for bread assay.

Pure pyridoxine HCl in 0.036*N* H_2SO_4 + 0.08*M* $NaC_2H_3O_2$ when put through the ion exchange step showed a recovery of 95%. Tripling the buffer concentration reduced the recovery to 82%.

It is convenient to prepare extracts of flour and bread and to carry out step (*a*) of digestion in the afternoon, then to carry out step (*b*) of digestion, ion exchange purification, oxidation, lactonization, and fluorometry determinations in the morning of the following day.

Results

The efficiency of the various steps in the analysis as determined by recovery experiments is as follows:

Extraction.—Pyridoxine HCl added to flour which was baked into bread undergoes no significant loss in extraction.

Digestion.—Pyridoxine HCl added following extraction and prior to incubation with the enzyme, Mylase P, is completely recovered.

Ion exchange purification.—The recovery at the ion exchange step may be reduced by acidities greater than pH 5 or salt concentrations greater than those recommended in the procedure. Even the natural mineral content of flour and the salt added to bread dough somewhat reduces the efficiency with which the pyridoxine is taken on the Decalso. The removal of the pyridoxine by 25 ml of 0.22*N* H_2SO_4 is completely efficient only if the Decalso is fully "activated" by the hot acid treatment.

Oxidation.—The conversion of pyridoxine HCl to pyridoxic acid is consistently 38–40% under the conditions given for this step.

Lactonization.—Pyridoxic acid lactone is produced in 95–98% yield from pyridoxic acid at this step.

Fluorometry.—If the Decalso has been adequately treated so as to remove all ex-

Table 1. Comparison of results of microbiological assay[a] for total vitamin B_6 (2) with results of fluorometric assay for pyridoxine

Sample	Mg Pyridoxine HCl Added per Pound	Vitamin B_6 as Pyridoxine HCl by Microbiological Assay	Added Pyridoxine HCl Recovered	Pyridoxine HCl by Fluorometric Assay	Added Pyridoxine Recovered
			Bread		
1–1	0.0	0.35		0.04 ± 0.05[b]	
1–2	1.5	2.13	1.78	1.61 ± 0.09	1.57 ± 0.10[b]
1–3	3.0	3.70	3.35	3.07 ± 0.21	3.03 ± 0.22
2–1	0.0	0.52		0.08 ± 0.04	
2–2	1.0	1.59	1.07	1.06 ± 0.08	0.98 ± 0.09
2–3	2.0	2.50	1.98	1.91 ± 0.16	1.83 ± 0.18
			Flour		
2–1	0.0	0.64		0.02 ± 0.05	
2–2	1.5	2.27	1.63	1.57 ± 0.11	1.55 ± 0.12
2–3	3.0	4.2, 3.5	3.56, 2.86	2.86 ± 0.20	2.84 ± 0.21

[a] The microbiological assays were conducted by J. M. Scheiner, Hoffmann-La Roche, Inc., Nutley, N.J.
The uncertainty reported for all data represents the confidence limit established from three determinations when P = 0.05 and is the product of the standard mean error times the Fisher value of t for this number of determinations and this probability. Nineteen of 20 additional triplicate determinations can be expected to give a mean falling within the confidence limit. For flour or bread containing 1-3 mg pyridoxine hydrochloride per pound, this confidence limit is of the order of ±8% of the triplicate assay average.

changeable iron, the solutions obtained by way of the ion exchange step will show the same transmission as the solutions obtained without the use of the ion exchange step (95–97% of that of pure water) in the analysis of pure pyridoxine HCl. There will be no turbidity in the solutions whose fluorescence is being determined unless the complexing solution is inadequate or the aluminum ion concentration is too high. The latter observation is evidence of incomplete "activation" of the Decalso.

The other enriching ingredients of flour and bread do not interfere in this analysis.

Acknowledgment

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Determination of Ethanol in Yeast-Fermented Liquors by Gas Chromatography

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The almost quantitative conversion of sugar into alcohol and carbon dioxide during yeast fermentation probably results from a series of reactions brought about by the metabolic activities of the yeast. During these reactions various intermediate products are momentarily formed and then used up in the succeeding stage of the process. Usually assays of alcohol content are made to determine the extent of fermentation.

Most assay methods in current use are time-consuming, tedious, or not specific. During the last few years gas chromatography has become established as a rapid, efficient, and relatively simple technique for separating and analyzing mixtures of volatile substances.

In the study reported here gas chromatography was used successfully to determine ethanol in fermentation liquids. A small amount of cell-free fermentation liquid injected into a chromatographic column produced a single large peak which was identified as ethanol.

The gas chromatographic method was compared with the AOAC refractometer procedure (1) for ethanol, since the latter is relatively rapid, simple, and widely used.

METHOD

Apparatus

Any conventional gas chromatographic apparatus may be used for this procedure. In our experiments, the gas chromatographic equipment was built in the laboratory (2). Thermal conductivity cells were employed for detecting substances emerging from the column.

Column.—The chromatographic column, constructed of five feet of ¼ inch copper tubing, was wound in the form of a helix about 6 inches in diameter, so that it could easily fit into a one gallon stainless steel Dewar flask. The inlet end of the column had two openings—one for helium gas, used as the carrier, and the other, covered with a rubber septum, for introducing the sample to be analyzed.

Column packing.—The column packing was prepared by dissolving 15 g β,β'-oxydipropionitrile in 240 ml acetone and mixing in 100 g of firebrick (60–80 mesh). The solvent was then evaporated under vacuum in a laboratory evaporator. The resulting mixture was free-flowing and easily packed into the copper tube.

Combustion train.—The outlet of the column was attached to a stainless steel combustion tube ¼ inch in diameter and 8 inches long which was filled with 20-mesh copper oxide and heated in a combustion furnace to 600°C. The outlet of the combustion tube was, in turn, connected to a 12 inch length of ¼ inch diameter glass tubing filled with magnesium perchlorate to remove water of combustion and water contained in the sample injected into the column.

Procedure

A series of fermented liquors was prepared using the quantities and ingredients listed in Table 1. For the determination of ethanol by the AOAC method, a fermented mixture was centrifuged to remove yeast cells and solids. One hundred ml of the supernatant was neutralized to pH 7.0 with NaOH and mixed with 50 ml distilled water. This mixture was then

Table 1. Ingredients for the preparation of fermentation mixes[a]

Formula	Sucrose	Salt	Brew Improver[b]	Malt	Yeast	Water
	(g)	(g)	(g)	(g)	(g)	(ml)
I	6.6	2.2	0.65	—	4.40	100
II	3.2	1.9	0.65	0.40	2.40	100
III	11.9	2.9	0.65	—	7.0	100

[a] Each mixture was heated in water-bath at 35°C for 6 hours with constant stirring.

[b] Consists of a salt mixture with the following composition: Ammonium monohydrogen phosphate, 4.0 g; potassium sulfate, 1.0 g; magnesium sulfate, 0.5 g; calcium carbonate, 1.0 g.

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

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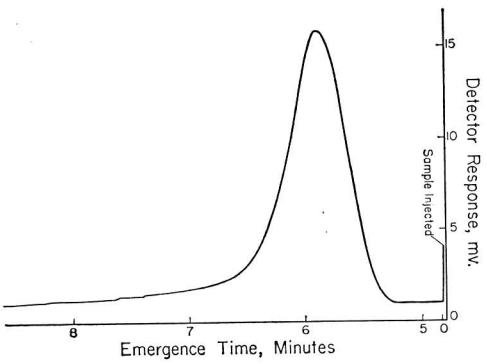

Fig. 1—Gas-liquid partition chromatogram of cell-free ferment.
β,β′oxydipropionitrile column ¼″ × 5 ft. at 75°; 85 ml He/min.; filament current 200 m.a.; 20 mv full scale deflection. Sample volume = 30 microliters.

distilled into a 100 ml graduated cylinder immersed in an air bath held at 25°C. The refractive index of the distillate was measured at 25°C with a Zeiss immersion refractometer. Percentage of ethanol by volume was read from refractive index tables and converted to percentage by weight.

For the determination of ethanol by gas chromatography, a weighed sample of the cell-free fermentation liquid (about 30 mg) was obtained by drawing approximately 30 microliters of solution into the barrel of a 50 microliter syringe. The syringe and contents were then weighed on an analytical balance, the sample was injected into the column, and the syringe was weighed once more to obtain the sample weight by difference. The column was maintained at 75° ± 0.5°C, and the flow rate of the gas (helium) was 80 ml/min. In all tests the chromatograms showed only a single peak (Fig. 1) which was identified as ethanol by comparison of retention volumes. With the type of column and detection system used in the present work, other alcohols that may be present in the fermented liquors apparently occurred in too small quantity to produce visible peaks on the chromatograms. Additional evidence of the identity of the peak was obtained by mixing ethanol with a fermentation filtrate, previously assayed, and observing the increase in size of the peak.

The recorder chart speed was maintained at 6 in./hr at the beginning of an analysis. Just prior to the issuance of the ethanol peak the chart speed was increased to 180 in./hr in order to obtain enlarged peaks whose areas could be accurately measured with a planimeter.

In gas chromatography, areas are frequently related to weight of sample. These areas are a function of chart speed, sensitivity of the recorder, retention volume of the sample, and column conditions during the experiment. Direct comparison between areas is difficult unless variables are strictly controlled. Thus an expression is needed which can provide a basis of comparison under all conditions. A peak-area term can be made to represent area in a generally applicable manner as follows:

Area of band in millivolts-milliliters = $K\ P\ S_n\ F\ /\ C$; where K = planimeter constant, P = planimeter reading of measured area in planimeter units, S_n = emf magnification factor (attenuation), F = flow rate of helium in ml/min, and C = chart speed in in./min.

To obtain the planimeter constant, K, where K = md/P, construct a rectangle on a section of chart paper and determine its area by means of the planimeter. Let d represent the distance in inches in the direction of chart travel and m the distance from the baseline, in millivolts, at an assumed attenuation of one, or full sensitivity of the recorder.

The linear relationships between the area measured and the concentration of ethanol measured by the above method are shown in Fig. 2. To test the efficiency of our measuring

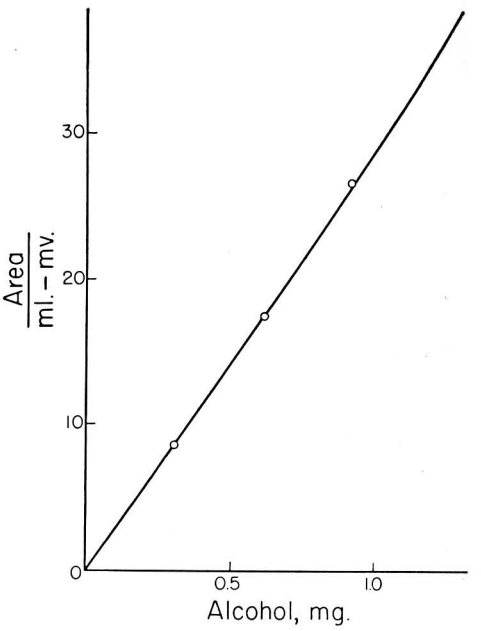

Fig. 2—Linear relationship between area under the curve and the concentration.

methods a solution of fermented liquid containing added ethanol was prepared in the following manner: 25 ml of a 92% ethanol solution containing 18.6 g ethanol was added to 475 ml of a cell-free ferment containing 24.13 g ethanol. The mixture containing the added ethanol was then assayed by both the AOAC refractometer and the gas chromatographic techniques.

Results

Table 2 shows the results obtained by analysis of the fermented liquor containing added ethanol, and Table 3 lists the results of ethanol determinations in a series of fermentation liquors by the gas chromatography and AOAC refractometer methods. In all tests the AOAC refractometer method produced higher values for ethanol than did the gas chromatographic method. These differences were probably due to measurements of other alcohols as well as ethanol by the AOAC method, whereas only ethanol was measured by the gas chromatographic method.

Table 2. Determination of added ethanol in a cell-free ferment[a]

Method	Alcohol in 100 g of Original Ferment	Alcohol in 100 g of Ferment Containing Added Ethanol		
		Found	Calculated	Recovery
	(g)	(g)	(g)	(%)
Gas chromatography	4.69	8.36	8.41	99.6
AOAC refractometer	4.82	8.50	8.54	99.4

[a] Formula 3, Table 1.

Both methods appear to have approximately the same precision and accuracy. Either method of assay accounted for 98% of added alcohol. The gas chromatography method, however, was much faster. A determination could be made every 10 minutes, whereas the AOAC method required at least one hour for each determination.

Table 3. The determination of ethanol in fermentation liquors by the proposed gas chromatographic method and the AOAC refractometer method

Sample	Fermentation Formula	Alcohol by: Gas Chromatography	Alcohol by: AOAC Refractometer Method
		(% w/w)	(% w/w)
A		2.39	2.50
B	I	2.31	2.71
C		2.38	2.74
Average		2.36	2.68
D		1.35	1.67
E	II	1.38	1.58
F		1.42	1.67
Average		1.38	1.64
G		4.75	5.00
H	III	4.85	5.03
I		5.01	—
Average		4.87	5.02

Summary

A rapid quantitative method for determining ethanol in fermentation liquors by gas chromatography is described and compared with the AOAC refractometer method now widely used. Both methods had the same precision and accuracy. However, the gas chromatographic technique was faster, specific, and required only very small amounts of sample.

Acknowledgment

The assistance of Dr. Walter S. Hale in the preparation of the various fermentation mixtures and the technical assistance of Dr. Fred Stitt are gratefully acknowledged.

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Volatile Alcohols in Dilute Aqueous Solutions by Alkoxyl Method

By CLARA McGREW and C. H. VANETTEN (Northern Regional Research Laboratory,* Peoria, Ill.)

A method of estimating trace amounts of ethyl alcohol (0.01 to 1.0 per cent) in aqueous solutions from alcohol-precipitated starch was required. Reported methods (1–8) were not well suited because of the presence of interfering substances and the need for special equipment or techniques. The alkoxyl method is used to determine alcohol of crystallization (9) and to analyze for ethyl alcohol in body fluids (4). These uses suggested extending this method to determining small amounts of alcohols in aqueous solutions. An additional advantage of the procedure was that estimation could be easily made with conventional alkoxyl apparatus, which is normally part of the equipment of a quantitative organic microanalysis laboratory.

Experimental and Procedure

The microalkoxyl method as described by Clark (10) was used with apparatus of essentially the same specifications as that recommended by the Committee on Microchemical Apparatus of the American Chemical Society (11). The reagent consisted of 5 ml 57% hydriodic acid (Merck HI [1] for methoxyl determination, sp. gr. 1.7; assay 55 to 58%). During preliminary runs in which the reaction time was varied, 1-hour distillation gave maximum recovery of ethyl alcohol as the volatile iodide. Carbon dioxide gas flow was 25–30 ml per minute, and temperature of the oil bath was 150–160°C. Water in the scrubber and the diluted hydriodic acid were changed after each analysis. With 1 ml of the solution under test containing 0.01 to 1.0% weight per volume of ethyl alcohol, titrations varied from 1.4 ml of 0.01*N* to 25.0 ml of 0.05*N* thiosulfate. A blank correction obtained from the reagents of about 0.20 ml of 0.01*N* thiosulfate was applied.

Dilution of the hydriodic acid solution by water in the sample was found to be the major factor preventing complete recovery of the alcohol as the volatile iodide. The relationship between dilution of the reagent and recovery of alcohol is shown in Fig. 1. The most likely explanation of lower recoveries is that as the hydriodic acid is diluted the reaction equilibrium between it and the alcohol to form the volatile iodide and water is shifted more in favor of the alcohol and hydriodic acid. Since the alcohols and alkyl iodides have similar boiling points, under these conditions some of the alcohol escapes before it has a chance to react with the hydriodic acid.

In obtaining the data for Fig. 1, 2–6.5 mg samples of the alcohol were introduced as 0.2–1.0% aqueous solutions. By using 0.25 ml of sample, or 10 ml of reagent and 0.5 ml of sample, recoveries close to 100% were obtained. For the problem at hand, 1 ml of sample and 5 ml of reagent was more convenient and used less reagent.

Results for a number of alcohols are shown in Table 1. No variation was observed in accuracy over the entire concentration range of 0.01 to 1.0 weight per volume per cent. Recoveries at concentrations below 0.1% were not as precise. The greater standard deviation obtained for ethyl alcohol was

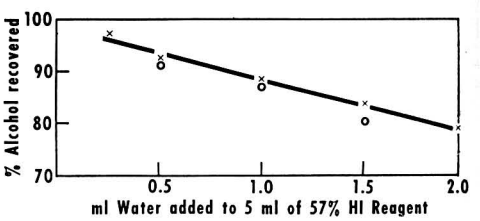

Fig. 1—Relationship of alcohol recovery as volatile iodide to dilution of hydriodic acid reagent with water.
X = Ethyl alcohol. O = n-Butyl alcohol. All points represent an average of two or more determinations. Line drawn through points obtained with ethyl alcohol.

* Laboratory of the Northern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

[1] Mention of firm names or trade products does not imply that they are endorsed or recommended by the Department of Agriculture over other firms or similar products not named.

Table 1. Analysis of solutions containing from 0.01 to 1.0 per cent weight per volume of various alcohols

Alcohol	Recovery,[a] Per Cent	Standard Deviation	No. of Determinations
Methyl	99.1	2.75	16
Ethyl	101.1	8.58	31
Isopropyl	97.1	5.45	16
Allyl	101.3	3.00	16
n-Butyl	104.9	5.34	15
Average	*100.72*	*5.59*	(94)

[a] These values were obtained by correcting the recovery of alcohol as the volatile iodide for the 12% loss indicated for the conditions used in the determination as shown in Fig. 1.

probably due to the fact that data were collected over about two years by two different operators using different sets of apparatus. Data for the remaining alcohols were obtained by one operator over a shorter period of time using one apparatus. Test solutions were prepared by weighing 10 g of reagent grade alcohols into small glass-stoppered Erlenmeyer flasks. The alcohol was diluted with water and made to 1 liter. Lower concentrations were prepared by proper dilution of this 1% solution.

Starch solutions containing no alcohol gave titrations of the same order as that of the reagent blank. Dissolved starch in the solutions did not interfere with alcohol recovery. Acetone gave no volatile iodides; the solvent dimethyl sulfoxide gave small amounts (less than 5%) calculated as SCH_3. These tests, made to detect substances that would interfere with the method, were run on 1% solutions.

Application

The alkoxyl method was successfully applied to estimating trace amounts of alcohols present in successive wash liquors from alcohol-precipitated starch. The absence of alcohol in the wash liquor showed that no additional alcohol was removed from the starch. A further application of the method might be the estimation of alcohols in hydrocarbon solvents after extraction into water, as suggested by Reid and Solmon (5). Additional applications, other than analysis of body fluids (4), might be the analysis of waste liquors from industrial processing and the detection of initial fermentation as evidence of spoilage. All alcohols that form volatile iodides could undoubtedly be measured by the procedure, except those such as tertiary butyl alcohol whose corresponding iodide is unstable.

Summary

The need for a method for estimating small amounts of alcohols in aqueous starch solutions led to a study of the microalkoxyl method. Dilution of the hydriodic acid reagent with water gave incomplete recovery of the alcohol as its volatile iodide. By correcting for this loss under standardized conditions the method successfully determined the amount of alcohol in aqueous starch solutions. Data are presented to show that the method can be used for measuring methyl, ethyl, isopropyl, allyl, and *n*-butyl alcohols present in aqueous solutions in concentrations from 0.01 to 1.0 weight per volume per cent. Based on 94 determinations of the alcohols the average recovery was 100.7% with a standard deviation of 5.59.

Acknowledgment

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Accuracy of Sugar Analyses of Honey by the Selective Adsorption Method

By JONATHAN W. WHITE, JR. and MARY H. SUBERS (Eastern Regional Research Laboratory,* Philadelphia 18, Pa.)

The selective adsorption method for determining sugars of honey (1, 2) has recently been adopted first action by the Association of Official Agricultural Chemists (3). In this procedure honey sugars are divided by charcoal column adsorption before analysis into three groups: monosaccharides, disaccharides, and higher sugars. Each fraction is analyzed for individual sugars by modifications of conventional volumetric methods.

In the development of the method, known sugar mixtures were subjected to the procedure and recoveries calculated. Additions of known sugars to honey solutions were satisfactorily accounted for.

The selective adsorption procedure has been used in the analysis of over 500 samples of honey from all parts of the United States. During this work opportunities were taken to obtain measures of the accuracy of the method. Aliquots of the three analytical fractions for each of 17 consecutive samples were evaporated and the dry weight was compared with that calculated from the sugar analyses. The results demonstrate the general accuracy of the method and also give some information about the materials not analyzed by the procedure.

The accuracy of the method as applied to honey monosaccharide fractions from the routine analyses of five honey samples was also checked by analyzing for dextrose and levulose polarimetrically as well as by the chemical procedure. While it has been shown (4) that polarimetric determination of fructose in honey is not accurate, charcoal column pretreatment removes interfering sugars and other materials and provides a solution containing only dextrose and levulose which can be analyzed polarimetrically.

Methods and Results

In the analytical procedure, the carbohydrates of a honey sample (0.8–1.0 g) are separated as follows:

Fraction A—250 ml–dextrose, levulose
Fraction B—250 ml–sucrose, reducing disaccharides
Fraction C—100 ml–higher sugars

The dextrose and levulose are determined individually. Reducing disaccharides are determined in Fraction B without preliminary hydrolysis and calculated as maltose; sucrose is determined by increase in reducing power after a mild acid hydrolysis. In Fraction C, reducing sugars after hydrolysis are determined by copper reduction and reported as dextrose.

Fifty ml aliquots of each of these three fractions from 17 consecutive honey samples were evaporated to dryness in a current of air on a steam bath and the weights of the residues determined. All solutions and residues were colorless. Table 1 shows the weights obtained for four representative

* Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

Table 1. Weight of material in 50 ml aliquots of analytical fractions

Fraction	Sample A Found (Mg)	Sample A Calc. (Mg)	Sample B Found (Mg)	Sample B Calc. (Mg)	Sample C Found (Mg)	Sample C Calc. (Mg)	Sample D Found (Mg)	Sample D Calc. (Mg)
Monosaccharide	140.3	138.9	136.8	137.6	135.0	134.7	132.1	132.2
Disaccharide	18.9	14.2	23.4	19.6	20.8	17.3	17.6	15.9
Higher sugars	7.0	5.6	10.0	8.2	9.7	6.9	10.5	8.0

Table 2. Analysis of variance for data in Table 1

Source of Variance	DF	Monosaccharides SS	MS	F	Disaccharides SS	MS	F	Higher Sugars SS	MS	F
Materials	16	896.8	56.0	2.86	163.1	10.2	11.2**	328.1	20.5	25.7**
Methods	1	2.18	2.18	0.11	58.8	58.8	64.9**	54.0	54.1	67.7**
Error	16	313.3	19.6		14.5	0.91		12.8	0.80	
Total	33	1212.28			236.4			349.9		

** Significant at .01 probability level.

Table 3. Percentages of material in analytical fractions, determined by two methods, whole sample basis

Fraction	Sample A By Wt.	Sample A By Anal.	Sample B By Wt.	Sample B By Anal.	Sample C By Wt.	Sample C By Anal.	Sample D By Wt.	Sample D By Anal.	Average 17 Samples By Wt.	Average 17 Samples By Anal.
Monosaccharide	71.37	70.67	69.68	70.12	67.82	67.70	69.97	70.03	71.23	71.06
Disaccharide	9.61	7.22	11.92	9.99	10.45	8.60	9.32	8.40	9.12	7.73
Higher sugars	1.62	1.15	2.16	1.68	1.95	1.38	2.22	1.70	2.18	1.22
Total sugars	82.60	79.04	83.76	81.79	80.22	77.68	81.51	80.13	82.53	80.01
Moisture[a]	15.7	15.7	15.8	15.8	18.2	18.2	18.0	18.0	17.3	17.3
Total	98.3	94.7	99.6	97.6	98.4	95.9	99.5	98.1	99.8	97.3
Not analyzed	1.7	5.3	0.4	2.4	1.6	4.1	0.5	1.9	0.2	2.7

[a] Moisture content of honey sample.

samples, together with the weight calculated from the chemical analyses. An analysis of variance on the individual weights of the three fractions from the 17 samples, found by weighing and calculated from the analytical values, is shown in Table 2. The difference in the results for Fraction A by the two methods is not significant, whereas the amount of unanalyzed material in both Fractions B and C is highly significant.

Table 3 shows the amount of material found in the fractions by evaporation and the amount calculated from the analyses. Both amounts were calculated for the entire sample. The last line [100 − (total material + water)] is the material not accounted for by each procedure. About 2.5% of honey material (17-sample average) in the three analytical fractions escapes analysis by the selective adsorption procedure. The distribution of this material among the three fractions is given in Table 4. It can be seen that the largest part of the material is in Fraction B—the disaccharides.

For the polarimetric determination of the sugars of Fraction A, 100 ml aliquots of Fraction A from five successive honey analyses were evaporated as before. They were made to 10.00 ml with water and a little

Table 4. Distribution of unanalyzed material on whole sample basis[a]

Fraction	Sample A	Sample B	Sample C	Sample D	17-Sample Average
Monosaccharide	0.70	−0.44	0.12	−0.06	0.40
Disaccharide	2.39	1.93	1.85	0.98	1.40
Higher sugars	0.47	0.48	0.57	0.52	0.52
Total	3.56	1.97	2.54	1.44	2.32

[a] Values show amount of unanalyzed material in each fraction, as per cent of entire sample.

ammonia, and their rotation was determined. The specific rotation was calculated using the evaporated weights, and the composition of the solution was calculated from the known values for pure levulose and dextrose. An example is as follows:

Sample E (Table 5). Original weight = 0.9958 g

Residue from 100 ml Fraction A = 0.2806g

Angular rotation (2 dm) = −1.55°

$[\alpha]_D^{20} = -27.62°$

$[\alpha]_D^{20}$ levulose = −92.5°; dextrose = 52.5°

$$\frac{-92.5 - (-27.62)}{-92.5 - (52.5)} = \frac{-64.88}{-145.0} = 44.74\% \text{ dextrose}$$

$0.2806 \times .4474 = .1255$ g dextrose

$.2806 \times (1 - .4474) = .1550$ g levulose

$$\frac{.1255 \times 2.5 \times 100}{.9958} = 31.51\% \text{ dextrose}$$

$$\frac{.1550 \times 2.5 \times 100}{.9958} = 38.91\% \text{ levulose}$$

Found by selective adsorption method:

31.19% dextrose,

39.15% levulose.

Table 5 shows the values so obtained for the five samples, and Table 6 shows an analysis of variance of these data. It can be seen that the variance is almost entirely due to materials (different honey samples) and that due to the methods is not significant at the 5% level for either dextrose or levulose. ($F = 6.4$ and 0.33; critical values at the 5% level = 6.39 for materials and 7.71 for methods).

Table 5. Determination of dextrose and levulose in monosaccharide fractions by two methods

Sample	Dextrose		Levulose	
	Chemical	Polarimetric	Chemical	Polarimetric
E	30.79	31.51	39.15	38.91
F	33.57	34.57	37.55	36.55
G	33.15	33.87	38.82	38.40
H	29.47	30.22	38.69	39.77
I	33.52	33.21	38.65	38.24
Av.	32.10	32.68	38.57	38.38

Discussion

The agreement between the values obtained by weighing and by calculation from the dextrose and levulose values in the monosaccharide fraction is satisfactory. This fraction is the most important in honey and makes up about 85% of the sugars. The 0.40% discrepancy found for the 17-sample average (Table 4) can be compared with the standard deviation obtained when four honey samples were analyzed by three analysts in one laboratory (0.38% for dextrose, 0.42% for levulose) (3).

The method of analysis for Fraction B is a compromise, since it has been found to contain maltose, isomaltose, turanose, maltulose, sucrose (5), and also kojibiose (6). There is also some evidence of trehalose (5) and leucrose (6). The relative reducing power of these sugars varies considerably; kojibiose is reported to have only about 6% of the reducing power of glucose toward the Shaffer-Hartman copper reagent (7). Trehalose, being non-reducing, would not be determined by the procedure used, but would appear in Fraction B if present. It is therefore likely that the un-analyzed material in the disaccharide fraction is at least in part

Table 6. Analysis of variance of data in Table 5

Source of Variance	*DF*	Dextrose *SS*	Dextrose *MS*	Dextrose *F*	Levulose *SS*	Levulose *MS*	Levulose *F*
Materials	4	26.08	6.52	48.5**	5.87	1.47	4.90
Methods	1	0.83	0.83	6.4	0.10	0.10	0.33
Error	4	0.52	0.13		1.19	0.30	
Total	9	27.43			7.15		

** Significant at .01 probability level. $F_{.05} = 6.39$ for materials; 7.71 for methods.

kojibiose; it may be seen from Table 3 to vary from sample to sample.

The un-analyzed material in Fraction C averages 0.52%. Inspection of the 17 samples shows that it does not vary as widely as does that in Fraction B. It may be a systematic error in the determination which is due to incomplete hydrolysis of higher sugars or destruction of fructose in the acid hydrolysis.

The essential accuracy of the analytical procedure is evidenced by the satisfactory agreement for dextrose and levulose values in the monosaccharide fraction by the two methods, plus the agreement between weighed and calculated residues. An earlier study of five methods of honey analysis made prior to development of the selective adsorption method (4) showed that variance due to methods was highly significant and greater than that due to differences among honey samples of different floral types. Here, Table 5 shows that variance due to samples is about ten times that due to methods in the analysis of monosaccharide fractions by two procedures (chemical and physical). Variance due to methods is not significant at the 5% level for either dextrose or levulose.

Summary

1. Comparison of dry weights of fractions from the selective adsorption analysis of honey with values calculated from the analysis shows that about 2.5% of the material passing through the charcoal column is not analyzed.

2. Most of this material is in the disaccharide fraction and probably represents kojibiose, and possibly also trehalose.

3. Polarimetric analyses of the monosaccharide fraction from the honey analyses gives results for dextrose and levulose not differing significantly from those obtained by chemical methods.

Acknowledgment

We wish to express our thanks to Mary L. Riethof who analyzed three of the honey samples.

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Chromatographic Determination of Hydrocarbons in Beeswax

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The hydrocarbon content of beeswax is a potentially useful analytical tool. Values for hydrocarbon appear in the literature from about 1890 (1). Two general methods have been most used, those of A. and P. Buisine (5) and of Leys (4). The former converted alcohols in the unsaponifiable fraction to acids by heating with potash-lime; hydrocarbons were the remaining petroleum ether-soluble material. In the Leys procedure, the hydrocarbons in the unsaponifiable fraction are separated by their insolubility in hot fuming hydrochloric acid-amyl alcohol. Vizern and Guillot (9) have proposed extracting the dry saponified wax with petroleum ether, and then acetylating the extract to remove small amounts of higher alcohols. All of these methods require considerable manipulation.

A chromatographic determination of paraffin-type hydrocarbons in carnauba wax, using alumina, is described by the ASTM (7). Curylo and Zalewski (3) have reported the analyses of 24 samples of beeswax from Poland, using chromatography on silica gel. Rosenberg (8) has described a chromatographic procedure for hydrocarbon content of waxes; he also used silica gel.

Newburger (6) described the use of alumina columns for separating hydrocarbons from cosmetic mixtures of beeswax and spermaceti. All material from a saponified mixture except hydrocarbon was found to be retained on the column. Using this work as a basis, Bruening (2) developed a quantitative procedure for determining hydrocarbon in cosmetic creams. He noted that it was not necessary to saponify beeswax before adsorption.

We have applied this procedure to the analysis of beeswax and mixtures of beeswax and microcrystalline wax. The volumes of solvents had to be modified slightly to obtain consistent hydrocarbon values for the microcrystalline waxes and mixtures. The method has been applied to the determination of hydrocarbon content of 60 samples of authentic beeswax and 20 samples of commercial microcrystalline wax. The complete results and analytical constants of the beeswax appear elsewhere (11).

METHOD

Materials

Alumina.—Alorco[1] Activated Alumina, Grade F-20, 80–200 mesh (Aluminum Company of America). Heat in an open pan in an oven at 190–200°C for 15 hours, cool somewhat in air, and store in closed containers.

Petroleum ether.—ACS grade, boiling range 30–65°C. Redistill if any interfering residue is left on evaporation.

Column.—A glass column, 16 mm o.d. × 300 mm with a 200 ml bulb on top and a Teflon stopcock on the lower end.

Pack the column as described by Bruening: place a pledget of cotton in the bottom and fill the tube with petroleum ether. Add the alumina to form a column 175 mm high, after light packing with about 5 pounds air pressure.

Procedure

Place 0.75 g sample (weighed to 0.1 mg) of beeswax (or sufficient sample to provide 100–125 mg hydrocarbon) in a 400 ml beaker, and melt on a steam bath. Add 250 ml petroleum ether, mark solvent level on outside, cover, and boil until wax is completely dissolved. Make volume to the 250 ml line, cool, and transfer the solution to the column. Regulate flow rate (gravity) to the fastest dropwise rate, collecting in a 400 ml beaker. Periodically transfer eluate to a weighed 50 ml beaker containing 6 glass beads and begin evaporating it on the steam bath. Do not allow beaker to cool after evaporation begins. After the sample is adsorbed, wash all residues into the column with 100 ml petroleum ether, then wash the column with 150 ml solvent (total

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[1] Mention of trade names in this paper does not imply endorsement by the U.S. Department of Agriculture over similar products not named.

volume 500 ml). Evaporate all eluate to dryness, place it under a heat lamp for 5 minutes, cool for 15 minutes, and weigh. Weight of residue divided by sample weight gives hydrocarbon content.

Freezing Point of Isolated Hydrocarbon.—This is most conveniently determined in a capillary. Capillary methods described in the literature for melting or freezing points of waxes (10) use open tubes, U-tubes, or closed tubes. Difficulty may be encountered when the tube, attached to a thermometer and immersed in a water bath, is heated. Frequently the sample may be displaced from the closed tube or may move upward in the open tube near the melting point. This point of movement is not reproducible, is not the melting point, and depends on the depth of immersion of the capillary and the depth of wax in the tube. Introduction of wax into the bottom of a closed tube is difficult. To eliminate these objections, let 3–5 mm of wax solidify in an open capillary and fill the remaining volume with water. (Proper positioning of the relative water levels in the tube and bath will allow the sample to remain in the tube after melting.) Fix the capillary to a thermometer graduated in 0.2° C divisions and immerse in a water-filled 25 × 110 mm test tube, then in turn immerse the test tube in a 1 L beaker filled with water. Stir the water by a slow air stream. Judge the freezing point of the hydrocarbon to be the first appearance of cloud in the melted sample.

Table 1. Reproducibility of chromatographic determination of beeswax hydrocarbon[a]

Sample	Hydrocarbon	
	Amount in Beeswax, %	Freezing Point[b], °C
45	13.68, 13.67	55.4, 55.1
47	15.31, 15.31	55.1, 55.1
48	14.32, 14.33	55.2, 54.8
49	13.64, 13.63	56.0, 55.8
50	14.80, 14.74	55.7, 55.4
51	14.30, 14.30	55.3, 55.2
52	14.60, 14.65	55.6, 55.3
53	14.11, 14.09	55.4, 55.3
54	15.48, 15.46	55.7, 54.6
55	13.73, 13.74	55.5, 55.0
57	13.67, 13.63	55.0, 55.0
58	15.02, 15.02	54.7, 54.5
59	14.04, 14.04	55.2, 55.2
61	14.92, 14.92	54.2, 53.9
62	15.46, 15.48	54.4, 54.3
s	0.016	0.17
C_v	0.11%	0.31%

[a] Each value is a single determination.
[b] These values are those of the individual samples.

Table 2. Determination of beeswax hydrocarbon by two analysts[a]

Sample No.	Hydrocarbon, %		Freezing Point, °C	
	Analyst A	Analyst B	Analyst A	Analyst B
3	12.29	12.61	56.0	55.9
4	17.09	17.21	53.8	53.4
6	12.92	12.95	55.9	55.8
22	13.82	14.08	55.6	55.6
s	0.15		0.15	
C_v	1.06%		0.27%	

[a] Each value is average of duplicates.

Results and Discussion

The reproducibility of the hydrocarbon values and of the freezing points of the isolated hydrocarbon may be seen in Table 1, where data from 15 consecutive determinations, in duplicate, are listed. The standard deviation of the hydrocarbon determination is 0.016% ($C_v = 0.11\%$) and for the freezing point determination is 0.17°C ($C_v = 0.31\%$). Table 2 shows the results obtained by two analysts for four samples run about six months apart. Here the standard deviation between analysts is 0.15% in hydrocarbon and 0.15°C in freezing point. Coefficients of variation are 1.06% and 0.27%.

Composition of the Isolated Hydrocarbon.—An infrared spectrum showed no structure other than that due to long-chain aliphatic hydrocarbon. The iodine numbers of two samples of beeswax hydrocarbon were 17.6 for Sample 41 (old comb wax) and 22.7 for Sample 43 (cappings wax).

Lewkowitsch (4) has reported beeswax hydrocarbons to melt at 49.5–59.5°, with iodine number of 20–22. Vizern and Guillot (9) report values for 12 preparations of 18.7–28.1.

Gas Chromatography.—The hydrocarbon fraction from a sample of old comb wax was subjected to gas-liquid partition chromatography. The column was an 8 ft. length of stainless steel tubing, 0.180 i.d., packed with "Chromosorb" 42–60 mesh coated with diethylene glycol succinate-adipate polyester (20%); column temperature was 225°C, helium carrier gas flow 40 p.s.i.g. Sample size

Table 3. Aliphatic hydrocarbons in beeswax

N-Hydrocarbon	Amount Present, %	
	Hydrocarbon Basis	Whole Wax Basis
Below C_{19}	trace	—
C_{19}	0.26	0.035
C_{20}	0.01	0.001
C_{21}	0.63	0.086
C_{22}	0.29	0.040
C_{23}	3.78	0.516
C_{24}	0.29	0.040
C_{25}	11.6	1.58
C_{26}	1.21	0.165
C_{27}	40.7	5.56
C_{28}	1.42	0.194
C_{29}	25.2	3.44
C_{30}	trace	—
C_{31}	14.5	1.98
C_{33}	trace	—

was approximately 1 microliter. Detector was a 4 filament thermal conductivity cell, current 200 milliamperes.

The results are given in Table 3. These data may be compared with the compilation given by Warth (10, p. 92), where the C_{31} hydrocarbon is reported in the highest concentration. Gas chromatography was not used to determine the values cited by Warth. It is noteworthy that the even-numbered hydrocarbons are definitely present, though at far lower concentrations than those with an odd number of carbon atoms. The log of the relative retention times of all hydrocarbons (including C_{20}, C_{26}, C_{28}, C_{32} added for identification purposes) fell on a straight line when plotted against chain length.

Summary

A rapid, reproducible procedure for the determination of hydrocarbon in beeswax and mixtures with other materials is described. Only hydrocarbons remain unadsorbed when a solution of beeswax in light petroleum ether is passed through a column of activated alumina. The freezing point of the isolated hydrocarbon is a useful constant for beeswax analysis. The beeswax hydrocarbons were analyzed by gas chromatography.

Acknowledgment

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Estimation of Microcrystalline Wax in Beeswax

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The detection and determination of mineral waxes in beeswax has been a problem for many years (1). Analytical constants for beeswax show considerable natural variation, so that although the presence of large amounts of non-reactive petroleum wax can easily be shown, use of these constants (saponification, acid, ester, and ratio numbers, and melting point) will not reliably indicate less than about 10% of such material in beeswax. The saponification cloud test has been proposed as a more sensitive qualitative test (2).

Examples from the literature of the ranges of values for these constants for yellow beeswax are given in Table 1. Their extent indicates their relative insensitivity for detecting admixture of hydrocarbons with beeswax. The hydrocarbon content is a more direct measure and has been proposed several times (5). Hydrocarbons are a natural constituent of beeswax; here again the natural variability must be established before reliable analyses can be made. Table 2 shows hydrocarbon values for beeswax from the literature. Of these, only Kebler and Bruening analyzed United States beeswax. Further data on the normal range of hydrocarbon content for domestic beeswax are needed.

* Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

We have determined the hydrocarbon content of 59 samples of crude yellow beeswax of known source from 20 states. In order to establish the authenticity of these samples, other analytical "constants" were also determined. These were melting point, saponification number, acid number, ester number, ratio number, and color. In the course of this work the freezing point of the beeswax hydrocarbon was also determined and found to be relatively constant. This value is much more sensitive to the addition of microcrystalline wax than is the melting point of the original beeswax.

MATERIALS AND METHODS

Beeswax Samples

Samples of ½ to 2 pounds of beeswax were collected from producers by the Bee Industries Association. Cappings wax and old comb

Table 2. Hydrocarbon content of beeswax

Value, %	Reference
10.4 –13.0	Leys (6)
12.7 –13.0	Buisine (7)
12.5 –14.0	Kebler (8)
12.5 –14.5	Buisine (9)
12.8 –17.3	Ahrens & Hett (10)
14.50–16.30	Vizern & Guillot (11)
13.6 ±0.48	Curylo & Zalewski (12)
14.93	Bruening (13)
12.28–17.09	This paper

Table 1. Standards for yellow beeswax

Pharmacopoeia[a]	Acid No.	Sapon. No.	Ester No.	M.P.	Ratio No.
U.S. (XV)	18–24		72–77	62–65°C	
German (DAB 6)	16.8–22.1		65.9–82.1	62–66.5	3.0–4.3
French	16.8–22.4	92–102	72–80	62–66	
British	17–23		70–80	62–64	3.3–4.2
U.S.S.R.	17–20.5		66–76	63–65	3.42–3.9
Govt. Spec.					
U.S.[b]	16.5–21.0	86–96		60.5–64.0	3.5–4.3
New Zealand[c]	17–21	87–103	70–80	62–64	3.3–4.2
TGA[d]	17–24	89–103	72–79	62–65	3.3–4.0

[a] From (3). [b] C.B.-191a (4). [c] N.Z.S.S. 743, 1950. [d] Toilet Goods Association, 1959 (in part).

Table 3. Analytical values for American yellow beeswax

Value	This Paper				Bisson, et al. (24)[a]			
	Mean	Range	s	C_v	Mean	Range	s	C_v
M.P.	63.56°	62.68–64.42°	0.35°	0.55%	64.1°	63.1 –65.0°	0.42°	0.65%
Acid No.	18.33	16.68–20.12	0.64	3.49%	18.6	16.8 –20.4	0.86	4.62%
Sapon. No.	90.94	88.62–94.39	1.35	1.38%	93.9	89.3 –99.3	1.84	1.96%
Ester No.	72.61	70.82–75.32	1.10	1.51%	75.3	71.1 –78.9	1.44	1.91%
Ratio No.	3.96	3.64– 4.31	0.15	3.79%	4.04	3.62– 4.59	0.19	4.70%
Hydrocarbon	14.59%	12.28–17.09%	0.76%	5.21%				
Hcbn. f.p.	54.9°	53.8 –56.0°	0.54°	0.98%				
Sapon. cloud test	62.5°	61.8 –64.6°	0.52°	0.83%				

[a] Their samples 43, 44, 45, 49 omitted (see text).

wax were collected, as well as four special samples, described later. The samples of old comb wax were accepted only from producers who could certify that the comb foundation used was known to be 100% beeswax, without admixture of strengthening materials used by some manufacturers. When samples were received at the laboratory, they were melted in porcelain on a steam bath and filtered through paper. Two samples had to be boiled in water to allow subsequent filtration. After filtration the wax was melted, stirred, and poured into approximately 8 × 12″ aluminum foil pans to a depth of 4–7 mm and allowed to cool; the sheets obtained were cut into 5–7 mm squares. This procedure assured representative analytical samples. Preliminary hydrocarbon analyses of samples taken from various parts of a larger (500 g) block of wax indicated that special measures were needed to insure homogeneity of samples.

Analytical Methods

Hydrocarbon.—This was determined by a modification (14) of the chromatographic method of Bruening (13). The freezing point of the isolated hydrocarbon was determined as previously described (14).

Saponification No.—After a preliminary study to improve precision of this determination as applied to beeswax, the procedure below was adopted. The methods of Grodman (15), the AOAC (16), Warth (17), Paquot and Perron (18), and a method described by Rosenberg (19) were studied. Some gave erroneously high results because of indicator malfunctioning in virtually non-aqueous systems, others showed poor reproducibility. The method below was found to be substantially that of ASTM D 1387–59 (20), with some differences. It was found that Kimble[1] glass flasks were quite satisfactory for this use, but since they are no longer commercially available, Pyrex and Kimax were tried and found unsuitable. Corning alkali-resistant Glass #7280 was satisfactory.

To a sample of 1 g beeswax in a 300 ml alkali-resistant flask, add by pipet (90 seconds drain time) 40 ml ca 0.2*N* alcoholic KOH (95% ethanol) purified as described by the AOAC (16). Boil the flask, with alundum boiling chips, 3 hours under water-cooled reflux on a steam bath, with occasional shaking. Remove flask, add 50 ml 0.1000*N* HCl by pipet, and reheat to the boil. Add 1 ml of 1% phenolphthalein, and titrate on a hot plate with magnetic stirring to disappearance of pink color. The solution must be near boiling at the end point for reproducible results. Run a blank at the same time.

Acid No.—Dissolve 2 g wax in 100 ml neutral 95% ethanol, boil on a steam bath for 3 minutes, and titrate on a hot plate, with magnetic stirring, with 0.2*N* alcoholic KOH, using 1 ml 1% phenolphthalein. Blanks are necessary.

Ester No.—Difference between saponification and acid numbers.

Ratio No.—Ratio of ester number to acid number.

Color.—Estimate by visual comparison of the solid wax in ca 5 mm thickness, with Munsell color chips (21).

Melting Point.—The ASTM method for melting point of paraffin wax (D87–57 (22)) was used, with the exception that the tempera-

[1] Mention of trade names does not constitute endorsement by the Department over others of a similar nature not named.

ture was measured with an iron-constantan thermocouple and recorded, and the sample was stirred. The procedure was calibrated with three known compounds freezing in the temperature range of interest, using a thermometer calibrated at the National Bureau of Standards. The determination is actually that of freezing point under strictly defined conditions. The ASTM procedure is used by some beeswax processors.

Flash Point.—Cleveland open cup, ASTM D-92 (23).

Saponification Cloud Test.—Federal Specification C-B-191a (4).

Results and Discussions

Table 3 gives a summary of the analyses of 59 beeswax samples, with the range, standard deviation, and coefficient of variation. Similar data are given also in Table 3 for 56 samples of Western U. S. beeswax, from the work of Bisson, Vansell, and Dye (24). Four of their samples (No. 43, 44, 45, 49) have been omitted from our calculations because they varied grossly in their characteristics from the remaining 56. They were "comb honey scrapings wax," which would be much higher in propolis (resins) than comb or cappings wax.

Table 3 shows that the characteristic that varies least ($C_v = 0.55\%$) among our samples is the melting point. It might be expected that this value would be most useful in characterizing the quality of wax or demonstrating admixture with higher-melting microcrystalline wax. Grout (25) has shown the effect of adding a microcrystalline wax—m.p. (drop point) 77°C—to beeswax—m.p. (drop point) 64.4°. He found that up to 10% of the former raised the drop point only to 65.0–65.6°C (about 1°C).

Table 4. Melting point of beeswax-microcrystalline wax blends[a]

Microcrystalline Wax, %		Melting Point, °C	Increase, °C
0		63.78	
5		64.63	0.85
10		66.00	2.22
20		68.68	4.90
50		72.72	8.94
100		78.22	
Beeswax, average[b]	$\pm 2s$	62.86–64.26°	
Beeswax, average	$\pm 3s$	62.51–64.61°	
Beeswax, average	$\pm 4s$	62.16–64.96°	

[a] ASTM Paraffin Wax Melting Point Determination.
[b] From Table 3.

Table 5. Flash points of waxes

Sample No.	Flash Point[a], °F
Cappings Waxes	
1	520
39	520
45	525
52	515
67	515
Old Comb Waxes	
8	490
17	510
23	510
54	505
73	520
Microcrystalline Wax[b]	570
70% Beeswax[c] 30% M.C. wax[b]	525
30% Beeswax 70% M.C. wax	540

[a] Cleveland open cup.
[b] Sample 18, Table 7.
[c] Sample 67.

Table 4 shows the melting points of several mixtures of beeswax and microcrystalline wax, determined by the same procedure used for the beeswax samples reported here. Also shown are intervals calculated from the data in Table 3—the $\pm 2s$ interval which should include 95.45% of beeswax samples, assuming a normal distribution; $\pm 3s$ which covers 99.73%; and $\pm 4s$ which includes 99.99% of samples. The 2.22° increase in melting point caused by addition of 10% of mineral wax is a bit greater than the $\pm 3s$ interval of 2.10°. It is less than the $\pm 3s$ interval (2.5°) calculated from the data of Bisson, *et al.* Thus, 10% addition of a sufficiently high-melting mineral wax might be detected by the melting point, using the apparatus described here.

The flash point has been suggested as a means of detecting mixtures of microcrystalline wax with beeswax. Little data on the flash point of beeswax appear in the literature. Stoeber in 1909 (26) gave the values for eleven samples of beeswax as

242–250°C (468–482°F), and that of most adulterants (at that time) as less than 200°C (392°F). Warth (27) gives the c.o.c. flash point of yellow beeswax as 468–482°F, and that of several microcrystalline waxes as 425–565°F. In Table 5 are given c.o.c. flash points of 5 samples of cappings wax and 5 samples of old comb wax, and those of a microcrystalline wax and two mixtures of the two. The beeswax values are higher than those reported by Stoeber and Warth, and addition of 30% of microcrystalline wax (flash point 570°F) raises that of the mixture only 10°F, so that it is still within the normal beeswax range. This determination is therefore not useful for this purpose.

The saponification cloud test is included in Federal Specifications for beeswax. It is required (4) that the temperature at which cloudiness begins be 65°C or below. Table 3 shows that the average temperature for the 59 samples is 62.5°C, with $s = 0.52°$. An interval of $\pm 4s$ gives 64.6° as the upper limit, within which only 1 in 16,667 genuine samples would not fall. The 65° limit set in the specifications is therefore quite liberal. Mixtures of 1, 2, 3, 4, 5, 10, and 20% microcrystalline wax and beeswax were tested. At 1%, the 61.8° cloud point of the beeswax was unchanged. The solution was slightly cloudy at the boiling point at 2 to 4%; at 5% and above, an insoluble layer at boiling was present; hence all except the 1% mixture failed the test. The 2 and 3% solutions were definitely cloudy when compared with controls.

The relative insensitivity of the common analytical "constants" of beeswax to admixture with inert waxes has been mentioned. By using the values for s for several determinations from Table 3, the minimum amount of inert material that could be detected through these analyses can be calculated. The sensitivity of this approach is limited by the extent of the interval representing normal variation in these "constants" in genuine beeswax. The fraction of genuine samples included within intervals of the mean $\pm 2s$, $\pm 3s$, and $\pm 4s$ (assuming a normal distribution) are shown in Table 6. Also the table shows the amounts of microcrystalline wax that could be added to a beeswax sample falling at the top of each interval without causing the value to fall below the stated lower limit. Thus, with interval limits of $\pm 3s$, one of every 370 samples of genuine beeswax would be expected to be rejected. Accepting this risk, at least 8% of mineral wax could be added to a beeswax with a saponification number at the top of the interval without bringing about rejection. To increase the sensitivity to 5.5% addition, one of each 20 samples of genuine wax would be rejected, an impractical situation. The situation is not actually as bad as this because samples showing high values for one constant do not necessarily also have high values for the others.

Table 6. Minimum amount of mineral wax detectable in beeswax by various determinations[a]

	Interval for Beeswax, in terms of s[b]		
	$\pm 2s$	$\pm 3s$	$\pm 4s$
Number of genuine samples in interval	19 of 20	369 of 370	16666 of 16667
Acid No.	14.0%	20.9%	27.9%
Sapon. No.	5.5%	8.25%	11.1%
Ester No.	6.0%	9.0%	12.1%

[a] Based on normal distribution.
[b] Data from Table 3.

This objection does not apply to the hydrocarbon determination, since the material added is determined. Here it is necessary to consider the apparent hydrocarbon content of microcrystalline waxes, determined as for beeswax. Twenty samples of commercial microcrystalline waxes as shown in Table 7 were subjected to the determination as used for beeswax; the freezing point of the isolated hydrocarbon was also determined. These results are shown in Table 7. The average value obtained for hydrocarbon [2] by the chromatographic procedures is 84.74%, with $s = 1.21$. The values ranged from 79.63 to 91.27%. Since the addition of 1% of an average microcrystalline wax will add 0.85% of hydrocarbon, this will increase the

[2] By "hydrocarbon" is meant the material passing through the alumina column in light petroleum ether solution.

Table 7. "Hydrocarbon" values of commercial micro-crystalline waxes

No.	M.P.[a], °C	Color[a]	"Hydrocarbon" %	"Hydrocarbon" f.p., °C
1	79.4–82.2	2 (ASTM)	91.27	67.1
2	—	Yellow[b]	84.29	78.6
3	71.1–76.7	White	84.65	70.8
4	—	Yellow[b]	87.67	76.2
5	81.1	Yellow 1¾ (NPA)	87.16	73.2
6	82.2–85.0	Amber 1½ (NPA)	80.13	78.7
7	73.9	White	88.13	73.1
8	76.7–79.4	White	87.42	72.9
9	73.9	Pale Yellow	84.96	72.8
10	82.2	Pale Yellow	81.95	78.6
11	79.4	White	85.11	71.2
12	78.3	Light Yellow	87.09	73.8
13	79.4–82.2	White	88.83	74.0
14	79.4	Amber[b]	79.63	74.4
15	82.2–85.0	White	86.83	74.8
16	76.7	Yellow	81.41	81.5
17	89.4	1¾ (NPA)	75.29	82.0
18	—	Light Amber[b]	87.61	73.8
19	—	Amber[b]	83.82	68.8
20	71.1–76.7	Amber	81.51	71.4
Mean			84.74	74.4
s			1.21	1.23
C_v			1.43%	1.65%

[a] As designated by manufacturer. [b] As estimated by authors.

hydrocarbon content of an average beeswax from 14.59 to 15.28%, producing a 4.73% change in hydrocarbon content.

It can also be calculated that at the $\pm 3s$ level $5.38\%\left(\frac{4.56}{.8474}\right)$ of an average microcrystalline wax could be added without raising the hydrocarbon content of a low-hydrocarbon beeswax above that of a high-hydrocarbon beeswax. Corresponding values for $\pm 2s$ and $\pm 4s$ are 3.58 and 7.16%. While this determination is somewhat more sensitive than those in Table 6, the freezing point of the hydrocarbon is still more sensitive. The average f.p. of the microcrystalline wax hydrocarbon fractions is 77.4°C, while that from beeswax is 54.9°C. The hydrocarbon isolated from mixtures shows freezing points between those of the two materials. The amount of increase in f.p. depends on the f.p. of the hydrocarbon of the particular microcrystalline wax present, and its amount. This is the reason for the variable per cent increase shown in Table 8 for the first four samples; the freezing points of the hydrocarbon from the microcrystalline waxes used were 82.0, 73.2, 67.1, and 68.8°C, respectively.

The intervals within which the hydrocarbon f.p. values for genuine domestic crude yellow beeswax can be expected to fall are

Table 8. Freezing point of hydrocarbon fraction of beeswax-microcrystalline wax blends

Composition % M.C. Wax[a]	F.P. of Hydrocarbon, °C	Increase[b], %
1	58.8	16
2	57.4	15
3	56.0	10
4	58.0	19
5	67.6	54
5	67.6	53
5	67.2	50
5	62.2	38
6	62.4	40
17	68.7	74
44	67.2	89
59	72.8	95

[a] Random pairings of 12 beeswaxes and 12 microcrystalline waxes from Tables 7 and 12.
[b] Expressed as per cent of difference beween f.p. of isolated hydrocarbon from specific waxes in each blend.

Table 9. Calculated ranges of freezing points of hydrocarbon fraction of U.S. yellow beeswax[a]

Interval	No. Included	Value, °C
$\pm 1s$	2 of 3	54.4–55.4
$\pm 2s$	19 of 20	53.8–56.0
$\pm 3s$	369 of 370	53.3–56.5
$\pm 4s$	16666 of 16667	52.8–57.0

[a] Assuming normal distribution.

shown in Table 9. The beeswax samples and the microcrystalline wax samples used to make these mixtures were selected and paired at random. It can be seen from Tables 8 and 9 that the presence of 5% of microcrystalline wax of the types described in Table 7 can be detected with a risk of rejecting only 1 genuine sample in 16,667.

Since both beeswax and microcrystalline wax vary in their hydrocarbon content as determined by alumina adsorption, calculation of the composition of a mixture from the hydrocarbon value found is at best an approximation. Table 10 gives the results of such analysis and calculation of composition of several mixtures. The samples of waxes used were all different, and selected and paired at random. The divergence between the known compositions and those found when calculations are based on the average values is of course wider than that calculated using the known hydrocarbon values for each member of the pair. The last two columns in Table 10 show the improved accuracy possible (s = 0.35 against 1.00) if the properties of the individual components are known.

The amount of microcrystalline wax in a mixture with domestic yellow beeswax may be estimated by the chromatographic determination of hydrocarbon content as previously described (14), and calculating:

$$\% \text{ Microcrystalline Wax} = 100\left(\frac{\% \text{ hcbn} - 14.59}{70.15}\right)$$

The standard deviation of the analysis, using these average constants, is 1% on the whole sample basis; if the hydrocarbon content of the components is known, this is reduced to 0.35%.

If the freezing point of the isolated hydrocarbon is 57°C or above (determined as previously described), the odds are 99,994 in 100,000 that it is not a genuine domestic yellow beeswax.

Data on Individual Samples.—Tables 11 and 12 show the physical and chemical data on the 63 individual beeswax samples. Samples 26–29, not included in the averages and calculations reported here, were produced at the Bee Culture Investigations Laboratory, Agricultural Research Service, Madison, Wisconsin. They were "scale" waxes, collected from caged colonies as follows:

	Rossman Hybrid Bees	*Starline Hybrid Bees*
Fed Sugar Sirup	No. 26	No. 28
Fed Clover Honey	No. 27	No. 29

None had access to pollen.

Summary

1. For 59 samples of crude yellow beeswax from the United States, the following average values were found, together with their standard deviations: melting point, 63.56°C (s = 0.35°); acid number, 18.33 (s = 0.64); saponification number, 90.94 (s = 1.35); ester number, 72.61 (s = 1.10); ratio number, 3.96 (s = 0.15); hydrocarbon, 14.59%

Table 10. Estimation of microcrystalline wax in yellow beeswax

Known Composition[a],	Composition Found			
	Using Averages		Using Individual Values	
%	%	Difference	%	Difference
0.93	1.78	+0.85	0.78	−0.15
2.12	1.92	+0.20	2.40	+0.28
3.14	3.35	+0.21	3.29	+0.15
4.02	3.76	−0.26	3.77	−0.25
4.79	4.09	−0.70	4.65	−0.14
4.54	4.58	+0.04	4.63	+0.09
4.79	4.68	−0.11	4.87	+0.08
4.88	3.43	−1.45	4.76	−0.12
6.07	6.81	+0.74	6.08	+0.01
17.49	16.39	−1.10	18.09	+0.60
43.60	44.89	−1.29	44.43	+0.83
59.23	61.30	+2.07	58.99	−0.24
Algebraic sum		−0.80		+1.14
s		1.00		0.35

[a] See footnote [a], Table 8.

Table 11. Physical properties of beeswax samples

No.	Origin	Type[a]	Color[b]	Melting Point, °C[c]
1	Columbia City, Ind.	C	5.0Y (7/6)	63.78
2	Galesburg, Mich.	OC	2.5Y (7/8)	63.45
3	Lowell, Mich.	OC	10.0YR (5/10)	63.58
4	St. Joseph, Mich.	OC	2.5Y (7/12)	63.08
5	Ft. Recovery, O.	C	2.5Y (7/10)	63.40
6	Wichita, Kan.	OC	10.0YR (4/4)	64.42
7	Pine City, Minn.	OC	2.5Y (7/8)	63.27
8	Shelbina, Mo.	OC	10.0YR (6/10)	63.10
9	Hamilton, Ill.	OC	10.0YR (4/6)	63.34
10	Streeter, N.D.	C	5.0Y (7/6)	63.50
15	Lake Leelanau, Mich.	OC	5.0Y (8/9)	63.83
16	Kent City, Mich.	OC	2.5Y (7/12)	63.63
17	Owosso, Mich.	OC	2.5Y (7/8)	63.27
20	Ortonville, Mich.	OC	2.5Y (6/10)	63.30
22	Taylor, Tex.	C	5.0Y (7/8)	63.83
23	Taylor, Tex.	OC	2.5Y (6.5/4)	63.50
24	Canton, O.	C	2.5Y (8/9)	63.36
25	Springfield, O.	C	5.0Y (7.5/8)	63.86
26	Madison, Wis.	S	White	62.92
27	Madison, Wis.	S	White	63.00
28	Madison, Wis.	S	White	63.18
29	Madison, Wis.	S	White	62.33
30	Chico, Calif.	OC	2.5Y (6/6)	63.34
31	Glenn, Calif.	C	3.5Y (7/8)	63.92
32	Orland, Calif.	OC	2.5Y (7/8)	63.88
33	Porterville, Calif.	C	5.0Y (6.7/4)	63.36
34	Modesto, Calif.	C	5.0Y (7/8)	63.36
35	Palo Cedro, Calif.	C	4.0Y (7/6)	63.51
36	Modesto, Calif.	C	2.5Y (7/8)	63.51
37	Tulare, Calif.	C	2.5Y (6/4)	63.84
38	Livingston, Calif.	OC	5.0Y (8/5)	64.27
39	Cumberland, Md.	C	5.0Y (8/8)	63.50
40	Red Springs, N.C.	C	5.0Y (8/6)	63.56
41	Rural Hall, N.C.	OC	2.5Y (6.5/8)	63.39
42	Glenarm, Md.	C	5.0Y (7/6)	63.78
43	S. Deerfield, Mass.	C	5.0Y (7.5/6)	63.92
44	Rural Hall, N.C.	C	5.0Y (7.5/8)	63.92
45	Lynchburg, Va.	C	5.0Y (8/5)	63.56
47	S. Deerfield, Mass.	OC	2.5Y (7.2/8)	63.43
48	Seville, O.	C	5.0Y (7.5/10)	64.21
49	Akron, O.	C	5.0Y (7.5/4)	64.24
50	Wellston, O.	C	5.0Y (7.5/7)	64.12
51	Elyria, O.	C	5.0Y (7.5/7)	63.92
52	Wapato, Wash.	C	5.0Y (7.5/6)	63.53
53	Livingston, Mont.	C	2.5Y (7.5/10)	63.50
54	Livingston, Mont.	OC	2.5Y (6/10)	63.30
55	Boise, Idaho	C	5.0Y (8/9)	63.86
57	Boise, Idaho	C	5.0Y (7.5/6)	63.93
58	Boise, Idaho	OC	2.5Y (7/6)	63.43
59	Durango, Colo.	C	5.0Y (7/12)	64.40
61	Wolf Point, Mont.	C	5.0Y (7.5/7)	63.61
62	Wolf Point, Mont.	OC	2.5Y (6/6)	63.03
63	Meeker, Colo.	C	5.0Y (11/7.5)	62.68
64	Meeker, Colo.	OC	10.0YR (7/12)	63.13
65	Marshall, Mich.	C	5.0Y (7.5/12)	63.41

(Continued)

Table 11 (*Continued*)

No.	Origin	Type[a]	Color[b]	Melting Point, °C[c]
66	Marshall, Mich.	C	5.0Y (8/9)	63.60
67	Cannon Falls, Minn.	C	5.0Y (8/7)	63.13
68	Paris, Tex.	C	7.5Y (8/8)	63.69
69	Monte Vista, Colo.	OC	10.0YR (4/6)	63.05
70	San Angelo, Tex.	OC	2.5Y (6/7)	62.85
71	Jeanerette, La.	C	5.0Y (8/9)	63.31
72	Tahlequah, Okla.	OC	10.0Y (5/8)	63.66
73	Garland, Tex.	OC	2.5Y (6/8)	63.60

[a] C=capping; OC=old comb; S=scales.
[b] Munsell Notation (21).
[c] Old comb waxes: Average 63.44°, Range 62.85–64.42°.
Capping waxes: Average 63.66°, Range 62.68–64.40°.
All waxes (except 26–29): Average 63.56°; $s=0.35°$; $C_v=0.55\%$.
Average of 26–29: 62.86°.

Table 12. Chemical Values of Beeswax Samples

No.	Acid No.	Sapon. No.	Ester No.	Ratio No.	Sapon. Cloud, °C	Hydrocarbon, %	Hcbn. f.p., °C
1	19.06	94.29	75.23	3.95	62.6	14.27	55.0
2	18.12	91.42	73.30	4.04	62.2	14.80	56.0
3	20.04	94.39	74.35	3.71	62.4	12.28	56.0
4	17.54	88.82	71.28	4.06	62.0	17.09	53.8
5	19.77	92.39	72.62	3.67	62.4	15.10	54.7
6	17.78	90.64	72.86	4.10	62.2	12.92	55.9
7	18.28	92.13	73.85	4.04	62.2	15.56	54.4
8	18.74	92.93	74.19	3.96	62.8	15.38	54.3
9	19.19	90.68	71.49	3.72	62.6	15.15	54.2
10	19.20	93.66	74.46	3.88	62.2	13.98	55.3
15	17.38	90.50	73.12	4.21	63.0	14.90	55.7
16	17.80	90.90	73.10	4.11	62.6	15.91	54.4
17	18.68	90.84	72.16	3.86	62.8	14.52	54.6
20	18.08	88.96	70.88	3.92	62.8	15.63	54.7
22	18.57	92.88	74.31	4.00	61.8	13.83	55.6
23	19.33	90.88	71.55	3.70	62.8	13.66	55.5
24	20.12	93.32	73.20	3.64	62.6	13.98	55.5
25	18.54	92.74	74.20	4.00	62.0	14.47	55.3
30	18.05	90.11	72.06	3.99	62.8	14.65	55.8
31	16.68	88.62	71.94	4.31	62.8	14.73	54.6
32	17.33	89.08	71.75	4.14	62.8	15.18	54.6
33	18.03	90.64	72.61	4.03	61.8	14.49	55.1
34	17.96	90.76	72.80	4.05	62.0	14.56	54.8
35	17.96	90.47	72.51	4.04	62.2	15.03	55.4
36	17.53	89.08	71.55	4.08	62.6	15.40	54.6
37	17.68	88.93	71.25	4.03	62.6	14.68	54.4
38	17.60	90.48	72.88	4.14	62.4	13.33	55.1
39	17.87	90.33	72.46	4.05	62.2	14.41	54.7
40	17.49	89.37	71.88	4.11	61.8	14.19	54.7
41	18.09	89.99	71.90	3.97	62.0	15.21	54.7
42	18.02	89.84	71.82	3.98	62.4	14.75	55.2
43	17.60	90.50	72.90	4.14	64.6	14.41	55.1
44	18.25	90.86	72.61	3.98	62.2	14.04	55.5
45	18.65	90.23	71.58	3.84	62.0	13.68	55.2
47	17.45	88.63	71.18	4.08	61.8	15.31	55.1

Table 12 (*Continued*)

No.	Acid No.	Sapon. No.	Ester No.	Ratio No.	Sapon. Cloud, °C	Hydrocarbon, %	Hcbn. f.p., °C
48	17.30	90.21	72.91	4.21	63.8	14.32	55.0
49	17.75	91.15	73.40	4.13	62.6	13.64	55.9
50	17.87	89.45	71.58	4.00	62.4	14.77	55.5
51	18.13	89.41	71.28	3.93	63.4	14.30	55.2
52	18.07	91.25	73.18	4.05	62.2	14.62	55.4
53	18.37	91.62	73.25	3.99	62.2	14.10	55.4
54	18.73	90.26	70.99	3.68	63.0	15.47	54.6
55	18.73	91.05	72.32	3.86	63.6	13.73	55.2
57	18.07	90.61	72.54	4.01	62.6	13.65	55.0
58	18.84	90.63	71.79	3.81	62.6	15.02	54.6
59	18.42	93.74	75.32	4.09	62.8	14.01	55.2
61	18.67	92.26	73.59	3.94	62.0	14.92	54.0
62	19.06	90.97	71.91	3.77	62.0	15.47	54.4
63	18.67	92.67	74.00	3.96	61.8	13.75	54.4
64	18.16	92.69	74.53	4.10	62.8	14.87	54.1
65	18.68	91.13	72.45	3.88	62.2	15.28	54.4
66	18.99	90.75	71.76	3.78	62.4	14.43	54.8
67	18.87	90.82	71.95	3.81	62.2	13.96	55.5
68	18.46	89.38	70.92	3.84	62.8	14.57	55.1
69	17.89	90.45	72.56	4.06	62.2	15.49	53.8
70	18.61	89.43	70.82	3.80	61.8	15.13	54.3
71	19.05	92.46	71.64	3.76	62.2	14.23	55.8
72	18.04	90.69	72.65	4.03	62.4	14.44	55.4
73	18.67	91.55	72.88	3.90	62.6	14.57	55.2
			Special Samples				
26	20.63	92.36	71.73	3.48	61.0	12.55	55.1
27	21.06	93.89	72.83	3.46	60.4	12.54	55.4
28	20.45	94.04	73.59	3.60	60.2	11.27	55.4
29	19.69	93.55	73.86	3.75	60.4	12.30	55.2
Av.	20.46	93.46	73.00	3.57	60.5	12.16	55.3
			25 Old Comb Waxes				
Av.	18.33	90.72	72.39	3.95	62.5	14.88	54.8
Range	17.30–20.04	88.63–94.39	70.88–75.32	3.70–4.21	61.8–63.8	12.28–17.09	53.8–56.0
			34 Cappings Waxes				
Av.	18.33	91.08	72.75	3.97	62.5	14.36	55.1
Range	16.68–20.12	88.62–94.29	70.82–75.23	3.64–4.31	61.8–64.6	13.64–15.40	54.4–55.9
			59 Cappings and Old Comb Waxes				
Av.	18.33	90.94	72.61	3.96	62.5	14.59	54.9
s	0.35	1.35	1.10	0.15	0.52	0.76	0.52
C_v	0.55%	1.49%	1.51%	3.79%	0.83%	5.21%	0.83%

(s = 0.76%); hydrocarbon freezing point, 54.9° (s = 0.54°); saponification cloud test, 62.5° (s = 0.52°).

2. The presence of 5% or more microcrystalline wax can be detected with certainty by the freezing point of the hydro-

carbon isolated in the chromatographic determination of hydrocarbon.

3. The microcrystalline wax content of a mixture with yellow beeswax may be estimated by determining the hydrocarbon content of the mixture.

4. The 65°C upper limit of the saponification cloud test is a reasonable value. This test will detect 2% of microcrystalline wax in domestic crude yellow beeswax.

Acknowledgment

We gratefully acknowledge the cooperation of the Bee Industries Association and many individuals in providing the beeswax samples; of Joseph A. Connelly in the instrumentation of the melting point apparatus; and of Marilyn K. Reader in the determination of some of the hydrocarbon values.

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Infrared Spectrophotometric Determination of Meprobamate

By WILLIAM R. MAYNARD, JR. (Virginia Department of Agriculture, Division of Chemistry and Foods, Richmond, Va.)

Meprobamate is the dicarbamate ester of 2-methyl-2-*n*-propyl propanediol. Other propanediol derivatives, such as mephenesin have been used as skeletal muscle relaxants. Available clinical evidence, however, indicates that meprobamate has an action of much longer duration. The drug appears to be useful as a mild hypnotic in simple insomnia or as a psychotherapeutic agent combined with psychotherapy in the management of psychoneurotic anxiety and tension states (1).

A number of methods have been proposed for determining meprobamate. Hoffman and Ludwig (2) use a colorimetric procedure in which meprobamate is reacted with *p*-dimethylaminobenzaldehyde and antimony trichloride in acetic anhydride to produce an intense red-violet color (2). This method, however, was designed for the determination of meprobamate in biological fluids in concentrations of from 0.5 to 10 mmg per ml. Another method, which is the official method in USP XVI, is based on a reaction that splits the meprobamate molecule into two fragments which can be determined by formol titration (3). Although this is a well-organized method, it is time-consuming and its specificity is somewhat questionable.

In the following method, meprobamate is extracted with chloroform. The absorbance at 6.32 μ is determined and compared with that of a given weight of a standard at this wavelength. All determinations were made with a Model 21 Perkin-Elmer infrared spectrophotometer. This offers a fast and accurate means of determining meprobamate in tables, capsules, and suspensions. The base line is easily established and the presence of stearates offers no problem since stearates do not absorb in the region taken for analysis.

Preliminary work indicated that reagent chloroform was the solvent of choice. Solubility estimates were made in order to determine what concentrations could safely be extracted, and the maximum amount that would be safely soluble for the purposes of this method was found to be approximately 10.0 mg meprobamate per ml chloroform. The optimum concentration selected for this analysis, however, is 4.0 mg meprobamate per ml.

A solution of meprobamate was found to follow Beer's law in concentrations of 2.0 mg to 10 mg per ml of chloroform, or to the approximate limits of its solubility.

In the case of the single drug, qualitative determination can usually be made at the same time the assay is performed by running the spectrum from 2.0 to 12.0 μ in chloroform solution and comparing with a standard meprobamate solution. If the medicament contains no other interfering drugs, the proposed USP XVI identification test can be used (3).

METHOD

Weigh 20 tablets or contents of 20 capsules and obtain average weight per tablet or capsule. Grind thoroughly in a mortar without appreciable loss and mix. Weigh an aliquot equivalent to about 100 mg meprobamate and transfer to a 25 ml volumetric flask. Make to volume with reagent chloroform and shake

Table 1. Experimental analysis of meprobamate

Sample No.	Meprobamate Taken (Mg)	Meprobamate Found (Mg)
1	50.0	48.2
2	75.0	77.6
3	100.0	103.0
4	120.0	118.2
5	140.0	144.6
6	160.0	162.0
7	180.0	176.0
8	200.0	204.0
9	210.0	212.4
10	220.0	218.0
11	230.0	225.2
12	240.0	236.0

frequently for 10 minutes. (For coated tablets, shake for 1 hour.) Filter, if necessary, through dry, fast filter paper, discarding the first 10 ml of solution, and read the absorbance of this solution with a double beam infrared spectrophotometer at 6.32 μ using 0.2 mm sodium chloride cells with a fixed slit width opening set at 150. Compare the absorbance of the sample with that of a standard and calculate mg meprobamate per unit.

Results

A series of known samples was analyzed. As shown in Table 1, the maximum percentage of error was not greater than 3.6% and the average percentage of error was less than 1.0%.

A number of commercial samples was then analyzed. Some of these samples contained an ingredient besides meprobamate. These other ingredients offered no interference in the determination (Table 2).

The method is fast and accurate and is applicable to tablets, capsules, and suspensions. Mixtures of other drugs will interfere only if they have an absorbance at the same wavelength used in the analysis.

Acknowledgment

We wish to thank Wallace Laboratories, New Brunswick, New Jersey, for supplying the pure meprobamate used in this study.

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Table 2. Analysis of commercial samples of meprobamate

Sample No.	Meprobamate[a] Guaranteed (Mg)	Other Ingredients in Preparation	Meprobamate Found (Mg)
1	400 per tablet	None	394.0
2	400 per tablet	None	406.0
3	400 per tablet	None	408.0
4	400 per tablet	None	406.0
5	200 per tablet	None	204.0
6	200 per tablet	None	202.0
7	200 per spansule	None	210.0
8	200 per 5 ml	None	210.0
9	400 per tablet	Dextroamphetamine sulfate	403.0
10	400 per tablet	Dextroamphetamine sulfate	420.0
11	200 per tablet	Pentolinium tartrate	209.0
12	200 per tablet	Pentaerythritol tetranitrate	195.0
13	200 per tablet	Promazine hydrochloride	208.0
14	200 per tablet	Prednisolone	206.0
15	200 per tablet	Conjugated estrogens	204.0
16	400 per tablet	Tridihexethyl chloride	403.0

[a] Each of these commercial samples falls within the tolerance of 5.0% allowed by USP XVI.

Determination of Gitaloxin in Digitoxin

By ALBERT E. H. HOUK (Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.)

The AOAC method (1) for the analysis of digitoxin preparations separates digitoxin from other glycosides by column chromatography. However, a new glycoside, gitaloxin, recently isolated from the leaves of *Digitalis purpurea* and identified as 16-formylgitoxin, has been reported by Haack, *et al.* (2) to constitute as high as 6% of commercial digitoxin preparations. Since gitaloxin has chromatographic and solubility properties similar to digitoxin, an initial study was made to ascertain whether there was any interference with the AOAC digitoxin assay, and to determine quantities present in USP grade digitoxin. It was found that gitaloxin is eluted quantitatively with digitoxin from formamide-Celite columns and that it gives about ¾ as much color with alkaline picrate as digitoxin does. Also, gitaloxin can be determined in the presence of digitoxin by an adaptation of Murphy's fluorometric procedure for gitoxin (3). A maximum of 2.6% gitaloxin was found in a preliminary survey of commercial digitoxin.

METHOD

Reagents

(a) *Propylene glycol-hydrochloric acid solution.*—Mix equal volumes propylene glycol and concentrated HCl, and store in refrigerator. Prior to use, warm the solution to 20°.

(b) *Gitoxin standard solution.*—1.00 mmg/ml. Dissolve 10.0 mg USP Reference Standard Gitoxin, $C_{41}H_{64}O_{14}$, in MeOH-$CHCl_3$ (1 + 2), dilute to 100 ml, and mix. Dilute 1.00 ml of this stock solution to 100 ml with MeOH-$CHCl_3$ (1 + 2), mix, and store all solutions in the refrigerator. Prior to use, warm the dilute gitoxin solution to 20°.

Apparatus

Photofluorometer.—Input filter with maximum transmission near 360 mμ, and output filter with maximum transmission near 470 mμ. Calibrate the instrument, using the reagent blank for zero setting and 2.5, 5.0, 7.5, and 10.0 mmg gitoxin standard, treated as directed below, for obtaining measurements in the optimum range of the scale.

Determination

Transfer 25.0 ml benzene-$CHCl_3$ eluate obtained from the formamide-siliceous earth column (1) and a suitable volume of gitoxin standard solution to separate small beakers. Evaporate just to dryness on steam bath with aid of air current. Cool, add 10.0 ml propylene glycol-HCl reagent to each beaker, and place in bath at 20° for 28 minutes. Mix frequently. Determine fluorescence of each solution, using reagent as blank, 30 minutes after addition of reagent. From the gitoxin standard and calibration line, calculate the % fluorescent substances, as gitaloxin, in the digitoxin sample.

Discussion

Gitaloxin, like gitoxin, produced a color with alkaline picrate reagent; its intensity, however, varying with specific conditions, was about ¾ that of digitoxin. It reacted with Keller-Kiliani reagent, but gave less than 1/20 the color of digitoxin with *m*-dinitrobenzene reagent (4). Highly purified digitoxin gave no fluorescence with propylene glycol-HCl reagent. Under the same conditions, gitaloxin and gitoxin gave similar strong fluorescence. Gitoxin is preferable to gitaloxin as a standard for determining gitaloxin since it is widely available and has been introduced as a USP Reference Standard.

The AOAC method for USP digitoxin (1), with the proposed modification for gitaloxin type compounds, was applied to purified digitoxin, a mixture of digitoxin and gitaloxin, USP Reference Standard Digitoxin, and five commercial USP digitoxin samples. Analytical data in Table 1 indicate that gitaloxin was eluted with digitoxin in the AOAC method of analysis. If present in appreciable quantity, it must be corrected for, and the correction can be determined by the proposed method. In agreement with Haack and collaborators (2), USP Reference

Table 1. Assay of digitoxin samples for gitaloxin-type compounds

Sample	Description	Digitoxin Fraction		Other Glycosides Fraction (%)
		Total Digitoxides (%)	Fluorescent Substances (Gitaloxin, etc.) (%)	
1	Digitoxin, purified	98.0	0.2	0.9
2	Digitoxin, purified-gitaloxin (9+1)	95.5	9.6	1.1
3	USP Reference Std. Digitoxin	98.0	0.0	1.9
4	Commercial Digitoxin A	91.0	0.7	4.6
5	Commercial Digitoxin B	92.0	0.6	4.8
6	Commercial Digitoxin C	86.3	2.3	10.2
7	Commercial Digitoxin D	91.3	2.6	7.8
8	Commercial Digitoxin E	94.3	2.2	6.3

Standard Digitoxin was found to contain no gitaloxin. In the five commercial digitoxin samples analyzed, gitoxin was still the chief contaminant, although three samples, all from the same source, contained over 2% gitaloxin.

Acknowledgments

Gitaloxin reference sample was obtained through the courtesy of E. Haack, C. F. Boehringer & Soehne G.m.b.H., Mannheim, Germany. Paper chromatography indicated the presence of small amounts of two contaminants, one more polar and one less polar than gitaloxin.

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Determination of Butylated Hydroxyanisole and Butylated Hydroxytoluene in Potato Flakes

By VICTOR J. FILIPIC and CLYDE L. OGG (Eastern Regional Research Laboratory,* Philadelphia 18, Pa.)

Anglin, Mahon, and Chapman (1) developed a method for the determination of butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) in edible fats. A modification of this method is satisfactory for the analysis of potato flakes containing these two antioxidants. Essentially, the method consists of determining the BHA by reaction with Gibbs' reagent (3), the total antioxidant content by the Emmerie-Engel method (2), and obtaining the BHT by difference.

In the Emmerie-Engel method, the phenolic antioxidants react with ferric chloride solution in the presence of 2,2'-bipyridine. The ferrous ion which results from reduction of the ferric ion by the antioxidant complexes with the 2,2'-bipyridine to give a characteristic red color. Gibbs' reagent (2,6-dichloroquinonechloroimide) couples with phenolics in the *ortho* or *para* position to form indophenols having a characteristic blue color. Consequently, BHA (2- and 3-*tert*-butyl-4-hydroxyanisole) will react, whereas BHT (3,5-di-*tert*-butyl-4-hydroxytoluene), being blocked in the reactive positions, will not.

Method Development

These phenolic antioxidants can be isolated from dehydrated potatoes by steam distillation of an aqueous slurry of the flaked product which normally is slightly acidic (about pH 6). However, sodium sulfite is added in the processing of potato flakes and the presence of sulfur dioxide in the distillate will interfere with both methods of analysis, causing low values by the Gibbs method and high values by the Emmerie-Engel procedure. Distillation from a medium sufficiently alkaline to retain the sulfur dioxide (pH 8–10) results in a positive interference in the BHA determination. The use of a scrubbing solution in the trap (Fig. 1) which is sufficiently alkaline to absorb the sulfur dioxide but not so basic as to remove the phenolics overcomes this problem. An aqueous suspension of magnesium oxide is recommended because it requires neither exact measurement of reagent nor adjustment of pH.

The reaction between BHT and ferric chloride is not completed in the time allotted by the procedure described; therefore accurate time control is necessary. The color development is also sensitive to temperature and light, so the sample and reagents must be adjusted to room temperature before mixing and the solution must be protected from light. The reaction between BHA, or a mixture of the two antioxidants, and ferric chloride behaves similarly. In addition, it appears that the presence of BHA accelerates the reaction rate of BHT without affecting the rate of BHA reduction of ferric ion. This conclusion is based on data (taken at constant temperature) presented in Table 1. In every instance, the absorbance of a solution containing both BHA and BHT is greater than the sum of the absorbances of each component. With BHA alone, Beer's law is obeyed, while with BHT alone, it is not. If,

Table 1. Absorbances of BHA and BHT solutions by the Emmerie-Engel method

	Mmg BHA				
Mmg BHT	0	4	12	20	*A*[a]
0	—	.026	.076	.128	—
4	.016	.058	.109	.161	.033
12	.052	.117	.174	.224	.095
20	.100	.182	.243	.290	.162

[a] Average absorbance of BHT in the presence of BHA, obtained by subtracting BHA absorbances (line 1) from absorbances for solutions containing both antioxidants.

* Eastern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture.

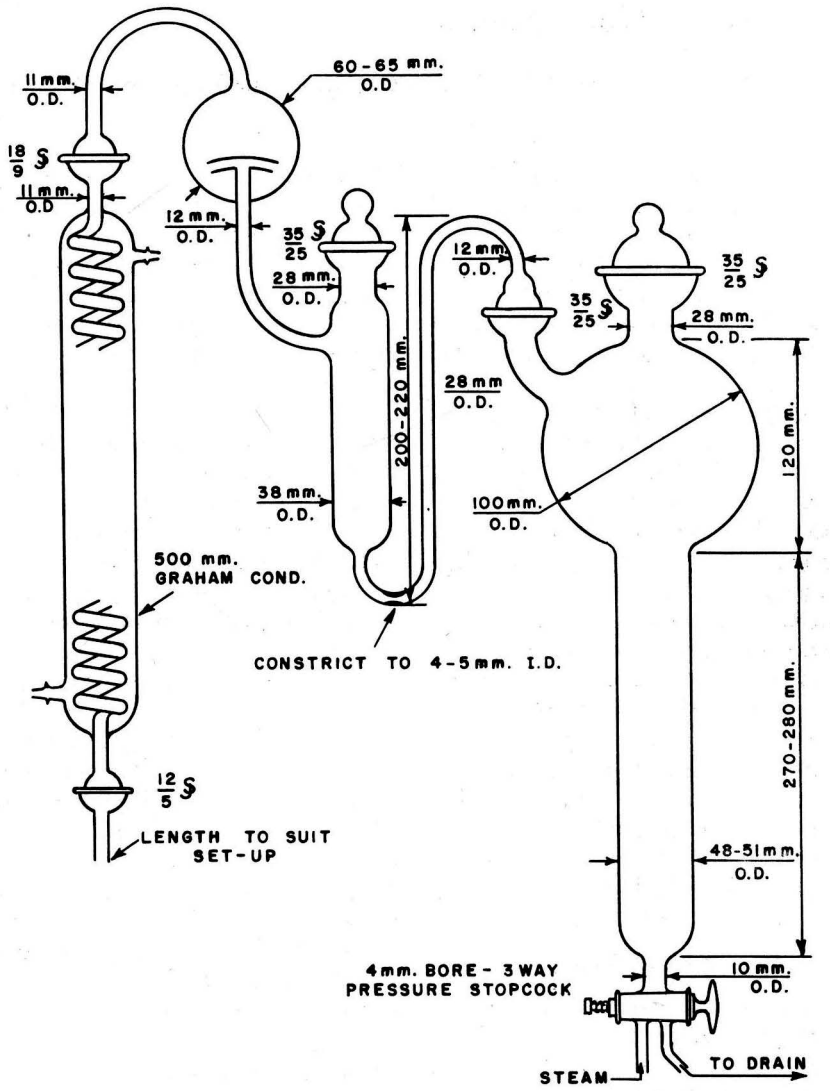

Fig. 1—Steam distillation apparatus.

in solutions containing both components, the absorbance due to BHT is calculated as the difference between the absorbance of the solution and that due to the BHA contained in the solution, Beer's law is then obeyed for BHT (last column, Table 1). The absorbance for BHT in the presence of BHA is almost twice that for BHT alone.

In the determination of BHA and BHT in edible fats (1), *n*-butyl alcohol is added to the reaction mixtures just prior to the measurement of absorbance to eliminate turbidity due to steam-distilled fat. A secondary effect is to inhibit strongly further color development, thus permitting more accurate absorbance readings. It is for this latter effect that butanol is employed in the procedure described below.

METHOD

Reagents

(a) *Ethyl alcohol.*—95% (or 2 to 1 methanol-isopropanol solution).

(b) *Magnesium oxide suspension.*—Place about 2 g MgO (reagent grade) in a 500 ml narrow-mouth Erlenmeyer flask. Add 200 ml H_2O and shake to create milky suspension. Shake again immediately before adding to scrubber.

(c) *Borax buffer.*—Dissolve 2 g $Na_2B_4O_7.10\ H_2O$ in 100 ml water.

(d) *2,6-D reagent.*—Dissolve 0.010 g 2,6-dichloroquinonechloroimide in 100 ml 95% ethanol. Prepare fresh every other day and store in amber bottle.

(e) *2,2'-Bipyridine.*—Dissolve 0.100 g 2,2'-bipyridine in 1 ml 95% ethanol and dilute to 50 ml with water. Prepare fresh daily. (If kept in amber bottle and stoppered when not in use, it can be used for 2-3 days.)

(f) *Ferric chloride.*—Dissolve 0.200 g in 100 ml water. Keep in amber bottle and prepare fresh daily.

(g) *n-Butanol solution.*—Mix 2 parts *n*-butanol with 1 part 95% alcohol (v/v).

Apparatus

(a) *Spectrophotometer.*—Instrument capable of accurately measuring absorbance at 515 and 610 mμ.

(b) *Still.*—Fig. 1; 35/25 stoppers are Ace Glass Co. No. 825.[1]

(c) *Steam generator.*—Electric pressure cooker with automatic control at 5 pounds steam pressure. (A Mirromatic[1] electric pressure pan with lid drilled and tapped for steam delivery tube has been found to be satisfactory.)

Distillation

Place about 135 ml of water in the still and add about 25 ml MgO suspension to the scrubber. Place a 250 ml volumetric flask, with mark at the 200 ml level, under the condenser tip. Introduce 20.0 $\pm$ 0.1 g potatoes into the still, stopper, and start steam flow immediately. Adjust steam flow to give approximately 20 ml condensate per minute. Distill 200 ml, turn off steam, and rinse condenser with about 40 ml 95% ethanol. Adjust flask to mark with 95% ethanol. Adjust distillate to temperature of standard solutions and start analysis within one hour.

Preparation of BHA and BHT Standards

Prepare a stock solution by dissolving 0.1000 g BHA in 95% ethanol; dilute to mark in a 100 ml volumetric flask, and mix. Prepare an intermediate solution by diluting a 10 ml aliquot to 100 ml in a volumetric flask with water. Prepare a standard solution by placing a 10 ml aliquot of the intermediate solution in a 250 ml volumetric flask, adding 190 ml water and bringing to mark with 95% ethanol. Prepare a standard solution of BHT in similar manner except that in preparing the intermediate solution, 50 ml of 95% ethanol must be added before dilution with water to keep the BHT in solution.

The standard solutions are stable for two days and the stock solutions for one week if they are kept refrigerated when not in use.

Analysis for BHA by Gibbs Method

Pipet 10 ml of about 20% ethanol into a small glass-stoppered bottle of 20 to 30 ml capacity and pipet 10 ml of distillate into a second bottle. Pipet 5 ml of about 20% ethanol and 5 ml BHA standard solution into a third bottle. Add 2 ml borax buffer, then 2 ml of 2,6-D reagent to each bottle and mix well. Measure the absorbance at 610 mμ in 1 cm cells *vs.* the blank in not less than 5 or more than 10 minutes after mixing. Calculate BHA content as follows:

ppm BHA = $A/B \times 25$, where A = absorbance of sample and B = absorbance of standard.

Analysis for BHT by the Emmerie-Engel Method

(Color reaction is light sensitive and consequently bottles used should be wrapped with black friction tape.)

Pipet 10 ml of about 20% ethanol into a taped 20 to 30 ml glass-stoppered bottle and pipet 10 ml of distillate into a second bottle. Pipet 5 ml of about 20% ethanol and 5 ml of BHA standard solution into a third bottle. Pipet 5 ml of BHA standard solution and 5 ml of BHT standard solution into a fourth bottle. Add 2 ml of the 2,2'-bipyridine reagent, then 2 ml of the ferric chloride reagent to each bottle and shake well. Let stand *exactly* 30 minutes, then add 5 ml *n*-butanol solution and mix well. *Exactly* 35 minutes after adding the ferric chloride reagent, start transferring the solutions to 1 cm spectrophotometer cells.

[1] Mention of a specific company or product does not constitute endorsement by the Department over other companies or products not mentioned.

Measure absorbance at 515 mμ of each solution *vs.* the blank at exactly 38 minutes after adding the ferric chloride reagent. Calculate BHT content as follows: *ppm* BHT = $\frac{C - (A \times D)/B}{E\text{-}D} \times 25$, where A and B = absorbances of sample and BHA standard, respectively, with Gibbs' reagent (above), C = absorbance of sample, D = absorbance of BHA standard, and E = absorbance of BHA–BHT standard.

The absorbance of the BHA standard by the Emmerie-Engel method need be determined only when a new reagent is prepared. The absorbance of the BHA standard by the Gibbs method and of the BHA plus BHT standards by the Emmerie-Engel method should be determined simultaneously with each pair of samples.

When first using the method, calibration curves may be prepared to make certain that standards, reagents, and apparatus are all functioning properly. A straight line plot should be obtained.

There is a tendency for the cells to adsorb color bodies from the solutions containing ferric chloride. For this reason, cell corrections should be checked frequently and the cells cleaned with dilute HCl periodically.

Results and Discussion

The reproducibility of analytical values was checked with two samples of commercial flakes. As presented in Table 2, the data show that BHA, BHT, and the total can be determined with a precision better than ± 1 ppm.

The efficiency of the steam distillation was also checked as follows: Twenty grams of potato flakes, which contained no antioxidants, were placed in the still and 130 ml water was added. Five ml of BHA and BHT intermediate solutions (0.5 mg BHA and 0.5 mg BHT) were added and steam distilled. Analysis of the distillates from three runs gave the following recoveries of antioxidants:

% BHA	*% BHT*	*% Total*
98, 93, 99	96, 93, 91	97, 93, 95

With flakes that had become slightly rancid it was necessary to remove interferences by steam distilling for two or three minutes before adding the antioxidants. Experience has shown that recoveries are significantly lower when more than one mg of antioxidant is present in the sample. Also, measurable blank values are obtained with untreated potato flakes and appreciable errors may occur at low levels of antioxidant. Analysis of potato flakes known to be free of antioxidants gave the following data:

BHA (ppm)	*BHT (ppm)*	*Total (ppm)*
0.3	2.8	3.1 (stored flakes)
0.7	1.9	2.6 (stored flakes)
0.0	2.0	2.0 (fresh flakes)

The method does not include corrections for blanks primarily because omission of the blank value approximately compensates for the small amount of antioxidants not recovered in the distillation step.

In this laboratory the house steam can be used for BHA and BHT analyses because it is sufficiently free of interferences. However, in some laboratories the use of house

Table 2. Antioxidant content of potato flakes

SAMPLE A			SAMPLE B		
BHA (ppm)	BHT (ppm)	Total (ppm)	BHA (ppm)	BHT (ppm)	Total (ppm)
26.4	10.3	36.7	25.5	6.9	32.4
25.9	10.7	36.6	24.7	7.6	32.3
26.2	11.5	37.7	25.0	7.1	32.1
26.2	11.5	37.7	26.0	5.6	31.6
25.7	9.9	35.6	25.7	6.5	32.2
25.0	11.1	36.1	25.7	7.4	33.1
25.3	11.1	36.4	25.7	7.4	33.1
Av. 25.8	10.9	36.7	25.5	6.9	32.4
S.D.± 0.5	0.6	0.8	0.5	0.7	0.5
Coeff. of Var. (%) 1.9	5.5	2.2	2.0	10.1	1.5

steam gave high BHT analyses. For this reason, an electric pressure cooker was adapted for use as a steam generator. Certain precautions should be observed in the use of a pressure cooker; it should be thoroughly cleaned and the rubber gasket should be soaked in alcohol prior to initial use. If the steam contacts rubber tubing high BHT values are likely to result; therefore a metal to glass connection must be made between the steam generator and the still.

It has been the procedure in this laboratory to give the still a special cleaning after about 25 analyses. Even though the still is rinsed after each analysis a residue from the potato flake samples builds up in time and can lead to significant blank values. This residue can be removed by back washing the still with a warm alcoholic KOH solution, followed by a dilute aqueous HCl solution, and finally rinsing with distilled water. The back washing or rinsing is done by allowing the suction created when the steam in the still condenses to draw the water or wash solutions backward through the still.

The presence of BHA appears to markedly reduce the stability of BHT in dilute solution. Consequently, standard solutions containing both BHA and BHT are not recommended because they are not stable and will lead to erroneously high BHT values when freshly distilled samples are analyzed. This instability is also the reason for specifying that sample distillates be analyzed within an hour after distillation.

Summary

A simple method for the rapid determination of BHA and BHT in potato flakes was developed by modification of a method applied to edible fats. The modifications consist of:

(1) The use of an alkaline scrubber to remove volatile acidic interferences during steam distillation of the sample.

(2) The determination of the absorbance of BHT in the presence of BHA.

(3) Use of a rapid steam distillation apparatus which eliminates the need for superheated steam or for applying heat to the sample container.

(4) Elimination of the use of absolute ethanol as solvent.

Acknowledgment

Dr. Robert L. Ferm of the Pillsbury Company and Mr. James Hale of the Borden Company provided the samples and participated in the collaborative study of the method. They obtained a similar order of precision in the analysis of BHA and BHT in these two samples. We are indebted to them, and to Dr. R. G. Buttery of the Western Utilization Research and Development Division, for testing the method and for suggesting refinements, particularly those concerning the stability of reagents and standards.

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Absorption Spectra of 1-Arylazo-2-Naphthol Food Colors

By W. PRZYBYLSKI and G. G. McKEOWN (Food and Drug Laboratory, Department of National Health and Welfare, Ottawa, Ontario, Canada)

Five coal-tar colors of the 1-arylazo-2-naphthol series used for coloring foods (1) are Sudan I (1-phenylazo-2-naphthol), Orange SS (1-*o*-tolylazo-2-naphthol), Oil Red XO (1-xylylazo-2-naphthol), Sudan R (1-*o*-anisylazo-2-naphthol), and Citrus Red No. 2 [1-(2,4-dimethoxyphenylazo)-2-naphthol]. Orange SS and Oil Red XO were formerly used in Canada and the United States for coloring oranges, cheese, and bakery products, but because of reports of chronic toxicity (2, 3), these colors were deleted from the permitted food color lists of both countries. Citrus Red No. 2, a new color which was developed as a replacement for Oil Red XO, is now permitted for use on oranges in both Canada and the United States. Sudan I and Sudan R are used to a limited extent in some European countries.

The objective of the present work was to determine spectral data for the above compounds over the 200–650 mμ region for analytical applications. Of particular interest were the effects of the different substituent groups on the absorption bands and the influence of solvents on the spectra as a means of providing more adequate characterization. Although a number of investigators have studied the spectra of these compounds, in most cases they reported only the wavelength and extinction coefficient of the main absorption band in the visible region.

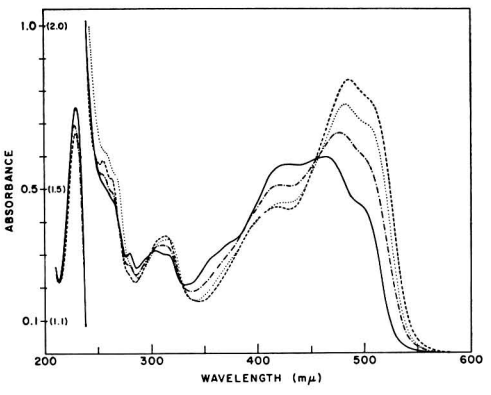


Fig. 1—Absorption spectra of Sudan I in hexane —; absolute ethanol _ . _ . _ .; aqueous ethanol _ _ _; chloroform ....

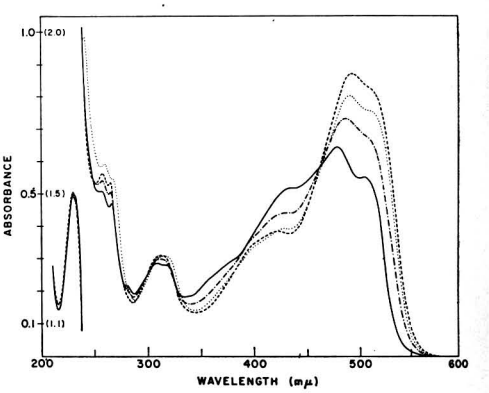
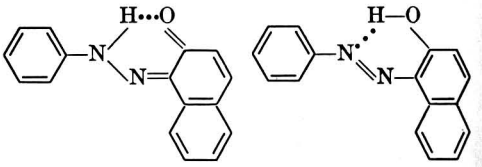


Fig. 2—Absorption spectra of Orange SS in hexane —; absolute ethanol _ . _ . _ .; aqueous ethanol _ _ _; chloroform ....

The structure of 1-arylazo-2-naphthol compounds was established by Burawoy, Salem, and Thompson (4) who showed from the electronic spectra of 1-phenylazo-2-naphthol and its derivatives that these compounds exist in solution as equilibria of internally hydrogen-bonded hydrazone (I) and azo (II) tautomers.



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These authors demonstrated that both the solvent and the nature and position of substituents on the phenyl ring influence the equilibria. The hydrazone tautomers are favored by polar solvents in the order 50% ethanol > chloroform > ethanol > hexane, and by substituents in the order *o*-MeO > *o*-Me > *m*-MeO > *m*-Me > *p*-Me > *p*-MeO.

The absorption spectra of Sudan I, Orange

SS, Oil Red XO, Sudan R, and Citrus Red No. 2 in the solvents hexane, chloroform, anhydrous ethanol, and ethanol-water (1:1,w/w) were determined. Figures 1–5 show some representative absorption curves, all of which were recorded at concentrations of 11.5 mg of color per liter (10 mm cell length). Molecular extinction coefficients were calculated for all significant points on the curves, and are presented in Table 1.

The spectra of all compounds in 50% ethanol show a strong absorption band (K-band) in the visible region which corresponds to the hydrazone tautomer (4, 5). A shoulder appears on the long wavelength side of these bands except in the case of Citrus Red No. 2 where the band is symmetrical. The maximum of this band shows a pronounced

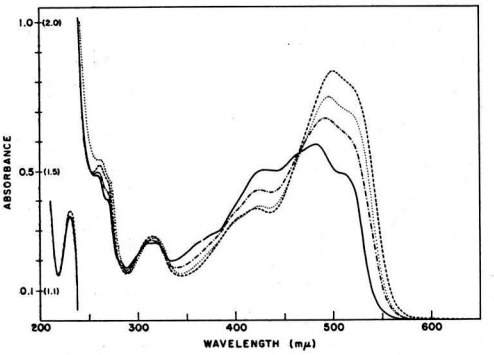

Fig. 3—Absorption spectra of Oil Red XO in hexane —; absolute ethanol _ . _ . _ .; aqueous ethanol _ _ _; chloroform . . . .

bathochromic shift (487–518 mμ) as the parent compound of the series is substituted on the phenyl ring in the order: H, 2-Me, 2,4 (2,5)-diMe, 2-MeO, 2,4-diMeO. It is unusual for a K-band to shift so markedly to longer wavelengths when substituents which do not appreciably increase the length of the conjugated system are introduced at non-terminal positions (6, 7). These band maxima therefore are only apparent; they arise from an overlap of the K-bands of the azo and hydrazone tautomers. Thus it is the shoulder on the long wavelength side of these bands that represents the K-band of the hydrazone tautomer. In the case of Citrus Red No. 2, the hydrazone tautomer predominates to such an extent in 50% ethanol that no false maxima appear. Resolution of the hydrazone K-band occurs in the spectra of Orange SS and Sudan R in hexane. Here the azo-hydrazone maximum has shifted to shorter wavelengths as a result of the increased contribution from the azo tautomer.

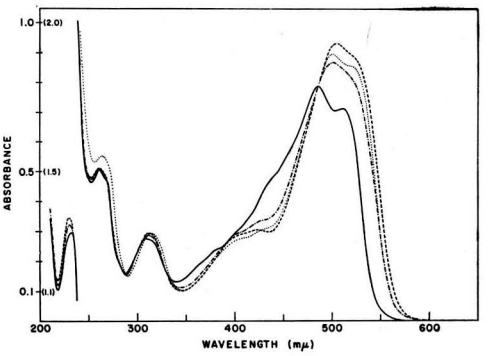

Fig. 4—Absorption spectra of Sudan R in hexane —; absolute ethanol _ . _ . _ .; aqueous ethanol _ _ _; chloroform . . . .

The effect of phenyl ring substituents on the azo-hydrazone equilibria is not additive in the case of Citrus Red No. 2. An *o*-MeO group favors the hydrazone tautomer in the case of Sudan R and a *p*-MeO group favors the azo species (4). In combination, the latter group does not cancel or diminish the effect of the former group but instead slightly augments its effect in favor of the hydrazone tautomer. A similar observation was made by Ospenson (8) in his studies on carboxyl-substituted 1-phenylazo-2-naphthols.

A K-band corresponding to the azo tautomer does not emerge as clearly as the

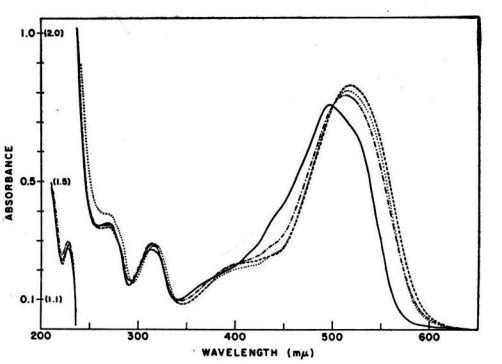

Fig. 5—Absorption spectra of Citrus Red No. 2 in hexane —; absolute ethanol _ . _ . _ .; aqueous ethanol _ _ _; chloroform . . . .

Table 1. Spectral data of Sudan I, Orange SS, Oil Red XO, Sudan R, and Citrus Red No. 2 in hexane, chloroform, absolute ethanol, and aqueous ethanol

Hexane λ(mμ) ε×10⁻³	Chloroform λ(mμ) ε×10⁻³	Ethanol λ(mμ) ε×10⁻³	Aq. Ethanol λ(mμ) ε×10⁻³	Hexane λ(mμ) ε×10⁻³	Chloroform λ(mμ) ε×10⁻³	Ethanol λ(mμ) ε×10⁻³	Aq. Ethanol λ(mμ) ε×10⁻³
Sudan I				Oil Red XO			
230		228	229	231		231	232
36.84		36.12	34.36	31.95		33.08	33.13
254*	255*	255*	255	258	261	260	262
11.0	12.7	12.7	12.58	11.73	13.03	12.70	12.98
263*	264*	264*	262*	270	271*	267*	267*
9.9	11.3	11.3	11.5	10.05	11.6	11.3	12.1
280	278*	278*	277*	281*		281*	
6.43	5.6	6.3	5.5	4.8		5.0	
303	311*			310			
6.62	7.1			6.20			
314*	314	310	313	316*	318	313	315
6.4	7.20	7.17	7.34	6.1	6.60	6.54	6.88
363*		376*		368*	380*	384*	381*
6.3		7.1		6.3	6.1	7.0	6.4
380*	381*	401*		383*	407*	406*	407*
7.5	6.6	9.5		7.3	8.20	9.3	8.5
430	426*	422	418	430	427	426	423
12.28	9.7	10.90	9.30	12.31	9.19	10.27	9.11
462	482	478	487	480	498	492	500
12.60	15.98	14.49	17.19	13.7	17.81	15.79	19.58
492*	499	495*	501*	508*	512*	510*	513*
9.6	14.86	13.4	16.2	11.3	16.9	14.6	18.6
Orange SS				Sudan R			
231		230	230	232		230	230
33.89		36.07	33.86	30.95		31.56	31.09
253*	260	257	257	261	263	261	260
11.5	13.37	13.08	12.84	12.10	12.96	13.10	12.82
266	267*	266	265	266*	270*	268	266*
10.62	12.4	12.32	12.19	11.5	12.3	11.78	12.01
279*	280*	280*		280*		282*	
5.1	4.9	5.0		5.0		5.4	
307				309			
6.61				6.59			
313*	317	312	313		313	313	312
6.5	6.95	7.39	7.18		6.87	6.54	6.51
363*				384*			
5.9				5.9			
383*	403*	402*	403*	407*	412*	408*	407*
7.2	7.9	8.93	8.1	7.5	6.8	7.2	6.8
438	431*	431*	422	443*	436*	432*	423*
11.87	9.0	10.69	9.10	11.9	7.5	7.8	7.1
477	491	489	493	486	500	509	505
14.57	18.24	17.77	20.29	18.74	21.05	20.86	21.08
504	509*	503*	507*	512	514*		517*
12.40	17.1	16.6	19.3	16.88	20.3		20.38

Table 1. (*Continued*)

Hexane λ(mμ) ε×10⁻³	Chloroform λ(mμ) ε×10⁻³	Ethanol λ(mμ) ε×10⁻³	Aq. Ethanol λ(mμ) ε×10⁻³	Hexane λ(mμ) ε×10⁻³	Chloroform λ(mμ) ε×10⁻³	Ethanol λ(mμ) ε×10⁻³	Aq. Ethanol λ(mμ) ε×10⁻³
Citrus Red No. 2							
227		228	229	396*			
33.40		34.26	34.09	5.3			
269	270*	269	270	423*	421*	423*	417
9.53	10.3	9.15	9.09	7.8	6.0	6.7	6.04
278*	280*	279*	280*	448*	443*	444*	442*
8.1	9.0	7.9	7.9	11.3	7.2	7.9	6.7
				497	516	514	518
				19.99	21.29	20.79	21.69
				518*			
				17.9			
312	316	313	315				
7.07	7.95	7.46	7.61				

* Denotes point of inflection.

hydrazone K-band in any of the spectra presented. However, the presence of the azo tautomer is indicated by the shoulder on the short wavelength side of the hydrazone band. Ospenson showed that this shoulder does not occur in the spectra of substituted 1-phenylazo-2-naphthol compounds that exist almost exclusively in the hydrazone form (8, 9). The spectra of Sudan I and Oil Red XO in hexane show a maximum for the azo tautomer at 430 mμ. Two low intensity bands that also appear to be related to the azo tautomer occur at about 360 and 375 mμ in all spectra taken in hexane. These bands occur in the spectra of the O-acetate and O-benzoate derivatives of 1-phenylazo-2-naphthol (5).

In the ultraviolet region a prominent absorption band occurs in all spectra at about 310–320 mμ. This band persists in acid solution (curves not shown). The location of this band remains remarkably constant in this series, despite substitution on the phenyl ring, for all the spectra taken in polar solvents in which the hydrazone tautomer is known to predominate. For example, the band maximum varies from 312 to 315 mμ in 50% ethanol. This behavior suggests that the band corresponds to the naphthoquinoimine moiety of the hydrazone structure; 1,2-naphthoquinone shows a strong ketonic band at approximately 330 mμ (10). In contrast, the spectra taken in hexane, where the absorption of the azo tautomer becomes appreciable, show the band to flatten and, in the case of Sudan I, to resolve at shorter wavelengths into a new band which is related to the azo tautomer. The O-acetate and O-benzoate derivatives of 1-phenylazo-2-naphthol both show a single band at about 300 mμ (5).

Another absorption band related to the azo tautomer occurs at 280 mμ. In 50% ethanol, all compounds display a minimum in this region, whereas in hexane and, to a lesser extent, in alcohol a small band appears which varies in intensity with the concentration of the azo tautomer. It appears most distinctly in the spectrum of Sudan I in hexane. It does not occur in the spectra of Sudan R and Citrus Red No. 2, both of which exist predominantly in the hydrazone forms in all solvents.

Two bands which appear to correspond in part to benzenoid absorption occur over the 250–280 mμ region in all spectra. The intensity and location of these bands are dependent on the relative concentrations of the azo and hydrazone tautomers and on the nature of substituents on the phenyl ring. Thus only those compounds whose azo-hydrazone equilibria vary with solvent show

a significant solvent effect. Citrus Red No. 2 and Sudan R, for example, show little solvent effect. The spectra of the remaining compounds show that these bands decrease in intensity as the polarity of the solvent, and hence the concentration of the hydrazone tautomer, decreases. Chloroform shows anomalous behavior, which is probably due to normal solvent effects. The effect of substitution on the phenyl ring on these bands is a bathochromic shift in the order of substituents H < 2-Me < 2,4 (2,5)-diMe < 2-MeO < 2,4-diMeO.

These spectra illustrate the importance of selecting the proper solvent when compounds in tautomeric equilibria are to be identified. Electronic spectra of closely related compounds such as structural isomers are often very similar if not indistinguishable. For example, one would expect that the hydrazones of *o-*, *m-*, and *p*-1-tolylazo-2-naphthols would exhibit very similar spectra. The same should apply to the corresponding azo isomers. The position of the methyl group should not be a major factor in determining the location and intensity of benzenoid and conjugation bands of these structures. In practice, however, solutions of these isomers each contain unique relative concentrations of both tautomers, the position of the equilibria being governed by the location of substituent groups and properties of the solvent. In hexane, for example, or ether (which is similar to hexane in its effect on these equilibria) the *para* isomer exists predominantly in the azo form, the *ortho* isomer exists in the hydrazone form, and the *meta* isomer shows intermediate behavior. In contrast, these isomers, when in a polar solvent such as 50% ethanol, all exist to approximately the same extent as the hydrazone form. Obviously, they can be distinguished most readily in hexane or ether. We propose to investigate the six isomers of 1-xylylazo-2-naphthol and report the results in a future communication.

Experimental

In these determinations the colors used were derived from commercial samples which were recrystallized from absolute ethanol to constant melting points which agreed with those in the literature. The values found were: Sudan I (1-phenylazo-2-naphthol), 135–137°C; Orange SS (1-*o*-tolylazo-2-naphthol), 132–133°C; Sudan R (1-*o*-anisylazo-2-naphthol), 183–184°C; Citrus Red No. 2 [1-(2,4-dimethoxyphenylazo)-2-naphthol], 159–160°C.

The sample of Oil Red XO, taken from a U.S. certified batch, was found to melt at 133-136°C. To determine its composition, the constituent isomers were separated by reversed phase paper chromatography (11), using Whatman 3 MM paper impregnated with 5% mineral oil in ether and developed by continuous descending flow with acetone-H_2O (1:1) for 72 hours. The colors were eluted from the paper and absorbances were read in the spectrophotometer. The sample consisted of approximately 7 parts of the 2,4-xylylazo isomer and 3 parts of the 2,5-xylylazo isomer. A preparative separation was also carried out by combining the eluates from a number of chromatograms. In this manner, a small quantity of each isomer was obtained in crystalline form, the melting points and spectra of which were found to be identical with those of authentic specimens prepared by synthesis.

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Studies on Coal-Tar Colors XXIV: FD&C Red No. 4

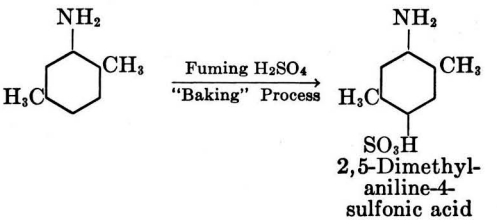
By JOHN A. WENNINGER, JOHN H. JONES, and MEYER DOLINSKY (Food and Drug Administration, Department of Health, Education, and Welfare, Washington, 25, D.C.)

The disodium salt of 2-(5-sulfo-2,4-xylylazo)-1-naphthol-4-sulfonic acid (Ponceau SX) is certifiable as FD&C Red No. 4 (1). The dye is prepared by sulfonating *m*-xylidine (2,4-dimethylaniline) with 20% fuming sulfuric acid (2) to give 2,4-dimethylaniline-5-sulfonic acid, which is then diazotized and coupled with 1-naphthol-4-sulfonic acid. Unpublished work in this laboratory has shown that some commercial samples of *m*-xylidine may contain significant amounts of the isomeric xylidines, 2,6-dimethylaniline and 2,5-dimethylaniline; therefore, it is possible that commercial FD&C Red No. 4 may contain the isomeric colors derived from sulfonated 2,6-dimethylaniline and 2,5-dimethylaniline.

This paper describes the preparation of two colors derived from the sulfonated derivatives of 2,6-dimethylaniline and 2,5-dimethylaniline. These colors are disodium salts of 2-(3-sulfo-2,6-xylylazo)-1-naphthol-4-sulfonic acid and of 2-(4-sulfo-2,5-xylylazo)-1-naphthol-4-sulfonic acid. Authentic samples of these colors were needed as standards in order to develop methods of separating them from FD&C Red No. 4.

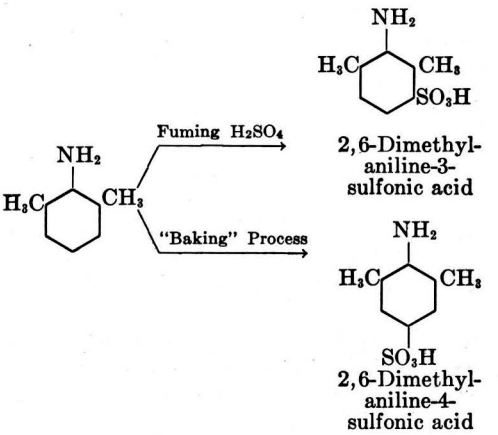
Experimental and Discussion

Direct sulfonation of aromatic amines with fuming sulfuric acid generally yields products in which the sulfonic acid group is meta to the amino group (3). Sulfonation by the "baking" process yields products in which the sulfonic acid group is para to the amino group unless this position is blocked (3). It is known (4), however, that sulfonation of 2,5-dimethylaniline by either the "baking" process or with fuming sulfuric acid yields the same product, which must be 2,5-dimethylaniline-4-sulfonic acid.



2,5-Dimethylaniline-4-sulfonic acid

We were unable to find any previous references to the sulfonation of 2,6-dimethylaniline. The expected products are:



2,6-Dimethylaniline-3-sulfonic acid

2,6-Dimethylaniline-4-sulfonic acid

Direct sulfonation of 2,6-dimethylaniline with fuming sulfuric acid.—To 214 grams of concentrated sulfuric acid, 100 grams of 2,6-dimethylaniline (Eastman "White Label") was added at a rate which maintained the temperature at 90°C. Sufficient 65% fuming sulfuric acid (118 grams) was added to the mixture at the same temperature to make the effective concentration of the fuming sulfuric acid 20%. After the addition of the acid, the reaction mixture was heated at 110°C for one hour, cooled, poured onto ice, filtered, and the product recrystallized from water. The yield after two recrystallizations was about 60 g. Titration of the product with standard sodium hydroxide

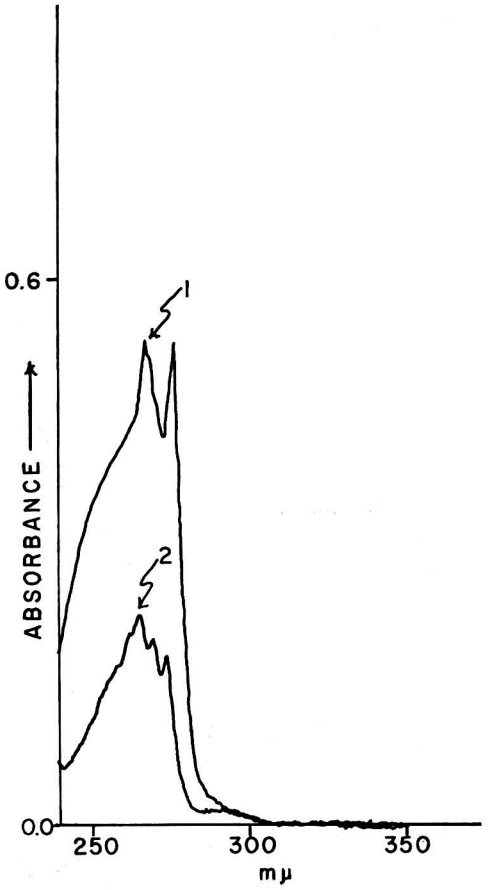


Fig. 1—Ultraviolet spectra: (1) 2,6-dimethylaniline-4-sulfonic acid, 100 mg/L in 0.1N HCl; (2) 2,6-dimethylaniline-3-sulfonic acid, 120 mg/L in 0.1N HCl. Cell length 1 cm.

solution confirmed the presence of a single sulfonic acid group. The equivalent weight calculated for $C_8H_{11}NSO_3$ was 201.2. The weight found was 200.7.

Sulfonation of 2,6-dimethylaniline by the "baking" process.—Equal molar quantities of 2,6-dimethylaniline and concentrated sulfuric acid were placed in a beaker and heated on the steam bath until a homogeneous mixture resulted. The reaction mixture was then heated in an oven at 200–230°C for six hours, cooled, mixed with ice, filtered, and the product crystallized from water. Fifty grams of 2,6-dimethylaniline yielded 31 g of product after two recrystallizations from water. Titration of the product with standard sodium hydroxide solution again confirmed the presence of a single sulfonic acid group. Equivalent weight calculated for $C_8H_{11}NSO_3$ was 201.2. The weight found was 200.9.

The ultraviolet and infrared spectra (See Figs. 1, 2, and 3) indicate that the products from the above sulfonations of 2,6-dimethylaniline are not identical. Based on previous work (3) it is concluded that the product obtained by the direct sulfonation with fuming sulfuric acid is 2,6-dimethylaniline-3-sulfonic acid and that obtained by the "baking" process is 2,6-dimethylaniline-4-sulfonic acid.

Sulfonation of 2,5-dimethylaniline.—2,5-Dimethylaniline was sulfonated by the "baking" process and with fuming sulfuric acid as described for the sulfonation of 2,6-dimethylaniline. The yields in each case were comparable to those given above. As expected, the two products obtained are identical. The compound undoubtedly is

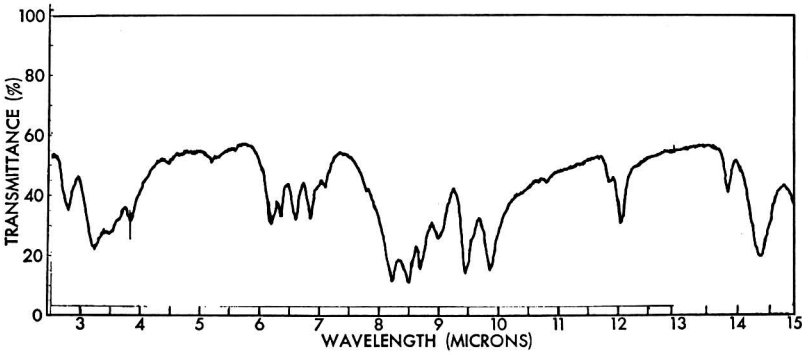


Fig. 2—Infrared spectrum of 2,6-dimethylaniline-3-sulfonic acid; KBr disk.

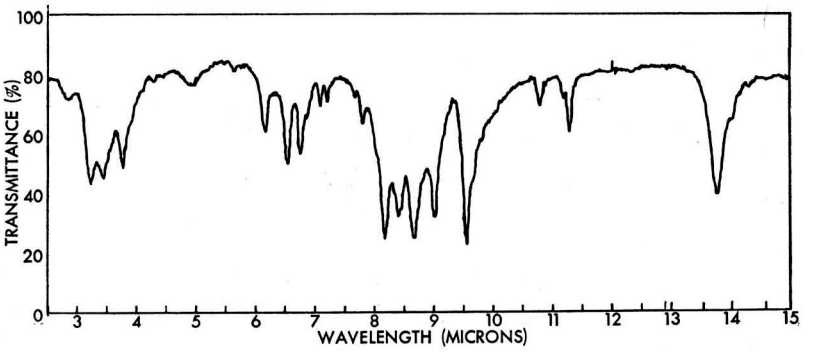


Fig. 3—Infrared spectrum of 2,6-dimethylaniline-4-sulfonic acid; KBr disk.

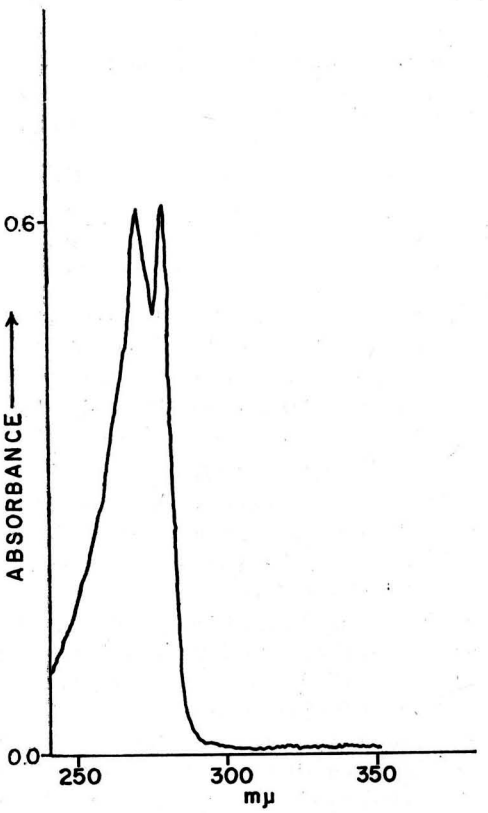


Fig. 4—Ultraviolet spectrum of 2,5-dimethylaniline-4-sulfonic acid, 100 mg/liter in 0.1N HCl. Cell length 1 cm.

2,5-dimethylaniline-4-sulfonic acid. The ultraviolet and infrared spectra of this compound are given in Figs. 4 and 5.

Since sulfonation with fuming sulfuric acid is the commercial method for the preparation of *m*-xylidine sulfonic acid, the products produced by this procedure are the only ones likely to be present in commercial *m*-xylidine sulfonic acid.

Preparation of Colors

The 1-naphthol-4-sulfonic acid was purified by crystallization of the *p*-toluidine salt of the acid (5). After several recrystallizations the melting point was 189°–190°C. [Literature, 190–192°C (5); 195°C (6)].

The 2,6-dimethylaniline-3-sulfonic acid and 2,5-dimethylaniline-4-sulfonic acid were then diazotized and coupled with purified 1-naphthol-4-sulfonic acid according to the method described by Graichen and Heine (7). The dyes were purified by dissolving them in a minimum amount of water and adjusting the pH to about 9. A saturated solution of NaCl in water was added to the dye solution until the dye began to salt out. After standing for three days at room temperature, the precipitated colors were filtered and dried. Analytical data are shown in Table 1.

CHROMATOGRAPHIC SEPARATION OF ISOMERIC COLORS FROM FD&C RED NO. 4

The following method was found suitable for separating 2-(3-sulfo-2,6-xylylazo)-1-naphthol-4-sulfonic acid from FD&C Red No. 4:

Reagents and Apparatus

(a) *Adsorbent.*—Powdered cellulose (Whatman Standard Grade).

(b) *Eluant.*—Dissolve 800 g sodium sulfate (anhydrous) in 5 liters 0.2*M* HCl.

(c) *Chromatographic tubes.*—Pyrex, 2-inch inside diam., about 24 inches long.

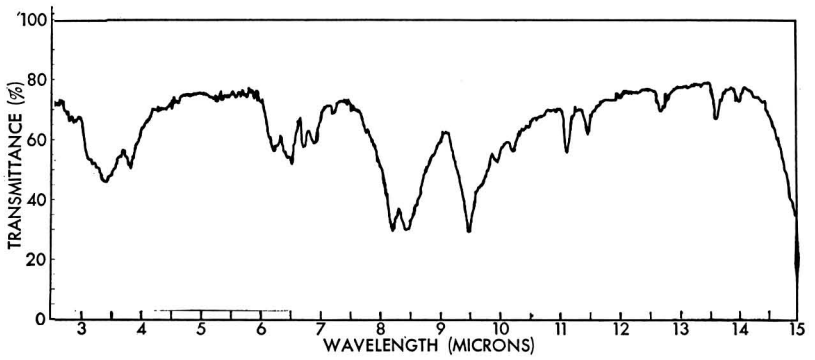


Fig. 5—Infrared spectrum of 2,5-dimethylaniline-4-sulfonic acid; KBr disk

Determination

Prepare a chromatographic column as described by Sclar (9). Carefully pipet 20 ml of a water solution containing 5 mg color/ml onto the top of the column, elute with the sodium sulfate solution, and collect the fractions containing the subsidiary color. (The isomer moves down the column more rapidly than does the FD&C Red No. 4.) Saturate the fractions containing the subsidiary color with sodium sulfate and pass the resulting solution through a small chromatographic column (1 inch inside diameter and about 15 inches in length) containing a 3-inch bed of powdered cellulose. Elute the color from this column with a small volume (about 100 ml) of water. Determine the concentration of the subsidiary color in the final eluate spectrophotometrically by comparison with solutions containing known amounts of the subsidiary.

Recoveries of isomer from mixtures of known composition are shown in Table 2.

Fifteen commercial samples of FD&C Red No. 4, including at least one sample from each manufacturer of the color, were chromatographed by the above method. Ten had no detectable amount of 2-(3-sulfo-2,6-xylylazo)-1-naphthol-4-sulfonic acid, three contained trace quantities, and two contained 0.5% of the isomeric color.

Table 1. Analyses of synthesized colors

	2-(3-Sulfo-2,6-Xylylazo)-1-Naphthol-4-Sulfonic Acid	2-(4-Sulfo-2,5-Xylylazo)-1-Naphthol-4-Sulfonic Acid
	(%)	(%)
Pure dye by titration ($TiCl_3$) (8)	91.1	89.3
NaCl	5.5	4.6
Volatile at 135°C (12 hours)	3.3	5.7

Table 2. Recoveries of isomeric color

FD&C Red No. 4	Isomer Added	Isomer Recovered
(mg)	(mg)	(mg)
96.0	4.0	3.3
99.0	1.0	0.7

Attempts to develop a chromatographic method for the separation of 2-(4-sulfo-2,5-xylylazo)-1-naphthol-4-sulfonic acid from FD&C Red No. 4 were only partially successful. Using Solka Floc (Grade BW-40) as the adsorbent and 20% Na_2SO_4 solution 1.0*M* in HCl as the eluant, a separation of 1 mg of the isomer from 19 mg of FD&C Red No. 4 was obtained after an elution time of about 40 hours. In this separation the isomer again elutes from the column ahead of the FD&C Red No. 4. Ten commercial samples, including at least one sample from each manufacturer of the color, were chromatographed by this procedure. None showed detectable amounts (detectable amount about 5%) of subsidiary.

Attempts to determine 2-(4-sulfo-2,5-xylylazo)-1-naphthol-4-sulfonic acid at the level of 1% in the presence of FD&C Red No. 4 were not successful.

Summary

Two colors, 2-(3-sulfo-2,6-xylylazo)-1-naphthol-4-sulfonic acid and 2-(4-sulfo-2,5-xylylazo)-1-naphthol-4-sulfonic acid, isomeric with FD&C Red No. 4, have been prepared.

Chromatographic methods for the separation of these colors from FD&C Red No. 4 are presented.

Samples of FD&C Red No. 4 submitted for certification were found to contain not more than 0.5% of 2-(3-sulfo-2,6-xylylazo)-1-naphthol-4-sulfonic acid and less than 5.0% of 2-(4-sulfo-2,5-xylylazo)-1-naphthol-4-sulfonic acid.

Acknowledgment

We wish to thank Mr. William B. Link of the Division of Cosmetics for supplying the purified samples of FD&C Red No. 4 used in this work.

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Separation and Identification of Eleven Organophosphate Pesticides by Paper Chromatography: Delnav, Diazinon, EPN, Guthion, Malathion, Methyl Parathion, Parathion, Phosdrin, Ronnel, Systox, and Trithion

By LLOYD C. MITCHELL (Division of Food, Food and Drug Administration, Department of Health, Education, and Welfare, Washington 25, D.C.)

Methods of analysis for residues of the organic phosphate pesticides on foods have been studied intensively in the Division of Food for several years. As one phase of this study, relatively pure solutions of a number of these pesticides were examined by paper chromatographic techniques, and a system of analysis has been worked out to separate and identify them, both singly and in mixtures. Preliminary cleanup and extraction procedures would, of course, have to be developed before the work reported here could be applied to routine examination of regulatory samples.

The eleven organophosphate pesticides for which separation and identification procedures are described are: (I) Delnav®,[1] (II) Diazinon®,[2] (III) EPN,[3] (IV) Guthion®,[4] (V) malathion,[5] (VI) methyl parathion,[6] (VII) parathion,[7] (VIII) Phosdrin®,[8] (IX) Ronnel®,[9] (X) Systox®,[10] and (XI) Trithion®.[11]

The compounds are located and identified in the chromatogram by (*a*) their quenched areas in ultraviolet light before and (*b*) after exposure to bromine fumes and spraying with fluorescein, (*c*) spraying with ammoniacal silver nitrate and exposure to the germicidal light, (*d*) spraying with 2-phenoxyethanol-silver nitrate and heating at 130–135°C for 30 minutes, and (*e*) exposure to the germicidal light. It should be noted that the (*c*) and (*d*) chromogenic agents listed above give more conspicuous spots if the chromatogram is first exposed, as in blueprinting, to the germicidal light.

The quenched areas produced in (*a*), above, can be reproduced for inspection in daylight by "printing" onto blueprint paper, but those in (*b*), above, have not been reproduced satisfactorily.

METHOD

Apparatus

(a) *Chromatographic tank and accessories.*—See *This Journal*, **36**, 1187 (1953); **40**, 999 (1957). A. H. Thomas Cat. No. 3677 is satisfactory.

(b) *Dipping tank and accessories.*—See *Ibid.*, **41**, 481, 781 (1958); A. H. Thomas Cat. No. 3680D is satisfactory.

(c) *Templates.*—See *Ibid.*, **43**, 748 (1960). Used to mark 8×8″ filter papers before spotting or washing.

(d) *Stainless steel Buchner-type funnel and accessories.*—See *Ibid.*, **43**, 748 (1960). Used to wash papers with water before chromatographing to remove, at least partially, substances which may interfere with the chromogenic agents.

(e) *Ultraviolet lamps.*—Model C-3, Chromato-Vue,[12] equipped with Model XX-15C, the long wave (3660 Å) ultraviolet lamp and C-81, the short wave (2537 Å) lamp; and the germicidal (2537 Å) lamp, equipped with two 36″ 30-watt germicidal tubes[13] mounted about 5″ above the chromatograms, and enclosed in a reflecting shield of sheet aluminum.

(f) *Chromatographic paper.*—Whatman No. 1 Filter Paper for Chromatography, 8×8″ sheets. Paper may contain impurities in sufficient quantities to interfere with chromogenic agents, and must be washed.[14]

[1] Hercules Powder Co., Wilmington, Del.
[2] Geigy Co. Inc., Bayonne, N.J.
[3] E. I. DuPont de Nemours & Co., Wilmington, Del.
[4] Chemagro Corp., Kansas City, Mo.
[5] American Cyanamid Co., New York 20, N.Y.
[6] Monsanto Chemical Co., St. Louis, Mo.
[7] American Cyanamid Co., New York 20, N.Y.
[8] Shell Development Co., Denver, Colo.
[9] The Dow Chemical Co, Holland, Mich.
[10] Chemagro Corp., Kansas City, Mo.
[11] Stauffer Chemical Co., Torrence, Calif.

[12] Chromato-Vue, Black Light Eastern Corporation, Bayside 61, Long Island, N.Y.
[13] General Electric 30-watt germicidal tubes.
[14] All papers used in this work were ruled with suitable template, washed with water at least 8 times in the metal Buchner-type funnel, and stored until needed in the cardboard box in which the filter paper was received.

(g) *Blueprint paper.*—Dietzgen, medium extra precoated with colloidal silica; Cat. #213, roll 36″ wide × 10 yards; speed HL.

Chromogenic Agents

(a)(*1*) Bromine, ACS grade. (*2*) 0.25% w/v, fluorescein in N,N-dimethylformamide (DMF) (Stock Solution). (*3*) Dilute 2 ml of (*2*) to 200 ml with 95% ethanol (Spray Solution).

(b) Dissolve 170 mg $AgNO_3$ in 1 ml H_2O, add 5 ml NH_4OH, and dilute with 95% ethanol to 200 ml.

(c) Dissolve 1.7 g $AgNO_3$ in 5 ml H_2O, add 20 ml 2-phenoxyethanol, dilute to 200 ml with acetone, analytical grade, and mix. (Should the solution show tendency to darken, add 1–2 drops of 30% H_2O_2, and mix. Even with addition of the peroxide, the solution may darken on standing, but the darkening apparently does not interfere with its chromogenic function.)

Samples

The samples used to prepare the standard solutions were labeled as follows: I, purified cis-trans isomers; II, pure, 99.2%; III, purified; IV, analytical standard; V, technical, 95%; VI, analytical standard, 80%; VII, technical, 98.5%; VIII, 63% alpha isomer, 32% beta isomer; IX, analytical standard, 99%; X, analytical standard, 26.1% (0.4 ml instead of 0.1 ml was used to prepare Solution A below); and XI, analytical standard.

Standard Solutions

(a) *Solution A.*—Dissolve 0.1 ml liquids or 0.1 g solids of each pesticide, and a mixture of all eleven pesticides, in separate 10 ml glass-stoppered volumetric flasks in ethyl acetate, and dilute with ethyl acetate to 10 ml. One lambda, or 0.001 ml, portions of Solutions *A*, the amount usually spotted, contain 10 mmg[15] quantities of the respective pesticides.

(b) *Solution B, C, D.*—If solutions are required which contain 5 mmg (*B*), 2 mmg (*C*), or 1 mmg (*D*) quantities of the pesticides in 0.001 ml, the aliquot usually spotted, then dilute 1 ml of Solution *A* of the respective pesticide(s) with 1, 4, or 9 ml ethyl acetate, and mix.

Nonaqueous Solvent Systems

(a)(1) *Immobile.*—Dilute 100 ml DMF (Industrial grade, Matheson Coleman and Bell Division, The Matheson Company Inc.) with ethyl ether, ACS, to 500 ml, and mix. (2) *Mobile:* 2,2,4-Trimethylpentane, pure grade (99 mole % min.) (Phillips Petroleum Co., Bartlesville, Okla.)

(b)(1) *Immobile.*—Dilute 50 ml formamide, reagent grade, with acetone, analytical grade, to 500 ml, and mix. (The solvent discolors on standing but apparently the discoloration does not interfere with its usage.) (2) *Mobile:* 2,2,4-Trimethylpentane.

(c)(1) *Immobile.*—Dilute 150 ml formamide with acetone to 500 ml. (2) *Mobile:* 2,2,4-Trimethylpentane.

Aqueous Solvent System

(a) *Immobile.*—Dilute 50 ml USP Heavy Mineral Oil with ethyl ether to 500 ml.

(b) *Mobile.*—Dilute 50 ml DMF with water to 100 ml, and mix.

One-Dimensional Procedure

Using a hard pencil and the template with 13 serrations, rule the starting line 1″ from the bottom edge of two papers for each solvent system, beginning 1″ from either side edge. (For this study, all papers were ruled, washed with water in metal funnel, *Apparatus* (d), dried at 100–110° C until dry (usually overnight, although the time depended on number of sheets washed), and stored several days before use.) Identify the 13 serrations as A, 1 to 11, A, respectively. Spot 0.001 ml portions of Solutions B (5 mmg) on all the numbered serrations, except 8. Spot Solution A (10 mmg) on 8 and Solution C (2 mmg of each of the eleven pesticides) on the two serrations marked A.

When the papers are spotted in this manner the chromatograms show the location of each compound; this in turn identifies some—not all—of the compounds in the mixture.

(a) *Nonaqueous systems.*—Add 50 ml of the mobile solvent, 2,2,4-trimethylpentane, to each trough in the chromatographic tank, and cover. Using a glass funnel, fill the dipping tank to within ⅛″ of the rim with the immobile solvent (20%, v/v, DMF in ethyl ether, or 10%, v/v, formamide in acetone), and cover. Attach a 2″ binder clip to the bottom edge of one paper, invert the paper, remove cover from the dipping tank, and insert the paper into the dipping tank until the solvent touches the starting line; immediately remove the paper, cover the tank, and clip the upper edge of the paper to a glass rod in

[15] For simplicity, 0.1 ml of liquid pesticide is assumed to weigh 0.1 g.

the hood. Repeat the dipping process for the second paper. As soon as the two papers have been dipped and clipped to the rod in the hood, reclip them to separate rods and transfer simultaneously to the chromatographic tank for development. Seal the glass cover with cellophane or masking tape (1″ wide). (Do the dipping and transferring in the minimum time.) Development time is about 75 minutes. If more than 6 or 8 papers are to be successively dipped, refill the dipping tank. As soon as the dipping is completed, drain the solvent from the dipping tank into a glass-stoppered flask by means of a glass siphon (do *not* pour); cleanse and dry tank.

(b) *Aqueous system.*—As described above, fill dipping tank to within ⅛″ of rim with the immobile solvent (10% v/v heavy mineral oil in ethyl ether), cover, and proceed to dip the papers. As soon as two papers have been dipped and clipped to rod in the hood, add 50 ml of the mobile solvent (50 ml DMF diluted to 100 ml with water) to each trough, reclip the papers to separate rods, transfer them simultaneously to the chromatographic tank for development, cover, and seal tank. Development time is about 90 minutes. Drain, cleanse, and dry tank.

Two-Dimensional Procedure

Using a hard pencil and the template, rule two papers for each pesticide. (For this study, all papers were ruled, washed with water, dried, and stored several days before use.) Use Solutions B, 5 mmg, and two papers for each pesticide. Spot 0.001 ml aliquot of the mixture (all 11 pesticides) at A (see Note) and one of the individual compounds at B (see Note). Spot as many pairs of papers as can be developed immediately, or until all 11 pesticides have been chromatographed individually at B. When papers are spotted in this manner, 8 or 9 of the 11 pesticides, depending on the chromogenic agent, can be identified by the compounds spotted at B and C.

Note: To clarify the directions for doing two-dimensional chromatography, or the discussion thereof, the three points at which to spot compounds on the paper are designated A, B, C. The dot in the lower left corner, 1″ from the bottom and left edges of the paper, is A, the point to spot the unknown samples (which should consist of three or more compounds) for two-dimensional chromatography. The serration on the line in the lower right corner, 1″ from the lower and right edges of the paper, is B, the point to spot the reference or known compound for the first solvent system. The serration on the line in the upper left corner, 1″ from the upper and left edges of the paper, is C, the point to spot the reference or known compound for the second solvent system. Further, to identify the unknowns resolved by two-dimensional chromatography, it may be advantageous to use 2 or 3 reference or known compounds; for example, spot different knowns ⅛″ on either side of the serration for two knowns; or on, and ¼″ on either side, for three knowns. Then the unknown compound, resolved by two-dimensional chromatography, which lies on or near the intersection of imaginary lines drawn parallel to the respective starting line from which the knowns migrated from B and C is the same compound as that applied at B and C.

The 5″ line near the upper part of the paper, parallel to the lower edge, marks the limit of the solvent front for the first mobile solvent system, while the 5″ line near the right edge of the paper, parallel to the left edge, marks the limit of the solvent front for the second system. The area between the two lines and A forms a 5″ square.

In practice, A, B, C are marked with a hard pencil to identify the sample or compound chromatographed.

(a) *First run: perpendicular.*—Add 50 ml of the mobile solvent, 2,2,4-trimethylpentane, to each trough in the chromatographic tank, and cover. Using a glass funnel, fill the dipping tank to within ⅛″ of the rim with the immobile solvent (20%, v/v, DMF in ethyl ether), and cover. Attach a 2″ binder clip to the bottom edge of one paper, invert the paper, remove cover from the dipping tank, and insert the paper into the dipping tank, until the solvent touches the starting line (points A and B); remove the paper, cover the tank, and clip the upper edge of the paper to a glass rod in the hood. Repeat the dipping process for the second paper. As soon as the two papers have been dipped and clipped to rod in the hood, reclip them to separate rods used in the tank, and transfer simultaneously to the chromatographic tank for development. Seal the glass cover with cellophane or masking tape (1″ wide). (Do the dipping and transferring in the minimum time.) At 5 minute intervals repeat the operation for tanks 2, 3, 4, etc., respectively. If more than 6 or 8 papers are to be dipped successively, refill the dipping tank. Let stand until the solvent front reaches the line ruled 5″ above and parallel to the starting points A

and B (about 45 minutes at 23° C). Immediately after completing the dipping process, drain the solvent from the dipping tank into a glass-stoppered flask, using a glass siphon (do *not* pour). Cleanse and dry tank.

As soon as the solvent front touches the 5″ line, break seal, lift cover, remove one paper, and restore cover. Transfer paper from glass rod used in tank to glass rod in hood. Repeat the operation for the second paper and let papers hang until dry (about 2 hours). While the papers are drying, remove solvent from troughs by means of 50 or 100 ml transfer pipet, applying suction mechanically (never orally). Then cleanse and dry troughs.

Transfer papers from rod in hood to a clean laboratory or spotting table so that C and A become the new starting points for the second solvent system. Using a capillary pipet, apply, at the serration designated C, 0.001 portions of the same standard solutions, respectively, as previously applied at serration B.

(b) *Second run: horizontal.*—Using a glass funnel, fill the dipping tank to within ⅛″ of the rim with the immobile solvent (10%, v/v, heavy mineral oil in ethyl ether), and cover. Attach a 2″ binder clip to the bottom edge (points designated C–A) of one paper, invert the paper, remove cover from the dipping tank, and insert the paper into the dipping tank until the solvent touches the starting points; remove the paper, cover the tank, and clip the upper edge to a glass rod in the hood. Repeat the dipping process for the second paper. Add 50 ml of the mobile solvent (50 ml DMF diluted to 100 ml with water) to each trough in the chromatographic tank, and cover. Reclip the two dipped papers to separate rods and transfer simultaneously to the chromatographic tank for development. Seal the glass cover with cellophane or masking tape (1″ wide). At 5 minute intervals repeat the operation for tanks 2, 3, 4, etc., respectively. If more than 6 or 8 papers are to be successively dipped, refill the dipping tank. Let stand until the solvent front reaches the line ruled 5″ above and parallel to the starting points C and A (about 75 minutes at 23° C). Immediately after completing the dipping process, drain the solvent from the dipping tank into a glass-stoppered flask, using a glass siphon (do *not* pour). Cleanse and dry tank.

As soon as the solvent front touches the 5″ line, break seal, lift cover, remove one paper, and restore cover. Transfer paper from glass rod used in tank to glass rod in hood. Repeat the operation for the second paper and let papers hang until dry (overnight). While the papers are drying, remove solvent from troughs by means of 50 or 100 ml transfer pipet, applying suction mechanically (never orally). Then cleanse and dry troughs.

Detection of Compounds in Chromatograms

(a) *By ultraviolet light.*—In the short wave light (2537 Å) compounds II, III, IV, VI, VII, and XI show as quenched areas in the dry chromatograms.

(b) *By blueprinting* (1,2).—(Use a dark room and keep light at minimum.) Lay chromatogram, front side up, on the sensitized surface of 9 × 8½″ blueprint paper so that the top edges of both papers coincide; clip upper corners together with small (No. 2) paper clips, cut off bottom edge of blueprint paper even with the chromatogram, using a paper cutter (12″ size is suitable), and clip lower corners together. Pencil the identification of the chromatogram on the back of the blueprint paper and smooth with fingers so that the two papers lie close together. Place paper on clean glass surface beneath the germicidal lamp, and place pieces of glass, 8×1″ (or similar size), on the two opposite edges of the overlapping blueprint paper to hold papers flat. Expose papers to germicidal light about 25 minutes (optimum time should be determined by operator). After exposure, wash blueprint paper in cold water by dipping it up and down into a filled sink, or holding it under the tap, until the spots are fully defined. Dry paper on clean flat surface, or clip to glass rod in hood. After drying, trim blueprint with paper cutter to size covered by the chromatogram. If blueprint is curled, flatten by rolling it in reverse direction, or store it under a weight overnight.

(c) *Chromogenic agents.*—Treat the odd-numbered chromatograms from each tank with the bromine-fluorescein agent, (a) *(1)(2)(3)*, as follows: Pipet a small quantity of bromine into a wide-mouth gallon jar, or other suitable glass container, and cover (1 ml will fume 16–20 chromatograms). Roll the chromatogram into a cylinder, uncover jar, drop cylinder into jar, cover, and fume about 30 seconds. Uncover jar, remove paper with tongs, cover jar, clip paper to glass rod, and within 30 seconds spray lightly (just dampen) with the diluted fluorescein. Within 3–5 minutes view paper under short wave ultraviolet light and circle spots with a soft pencil.

Bromine changes fluorescein to eosine and

causes the paper to appear light pink in daylight. Under the short wave light, the pesticides appear as quenched areas (deep, dark blue, sometimes almost black) against a background of greenish yellow (aqueous system) to bright yellow (nonaqueous systems). If the fluorescein is not changed by the bromine to eosine, there is little or no contrasting background and the spots may be masked or obliterated. DMF, or formamide, also interferes with the reaction; hence the chromatograms must be air dried (3–4 hours for the nonaqueous systems, and usually overnight for the aqueous system) before bromination and spraying with fluorescein. Those papers developed with the aqueous solvent system (nonvolatile mineral oil) may show poor contrasting background. Neutral fluorescein works better than either alkaline (NaOH) or acid (HCl) solutions.

Treat the even-numbered chromatograms from each tank with the ammoniacal silver nitrate agent as follows: After spraying the chromatogram with ammoniacal silver nitrate, expose at once to the germicidal light, first the back side, then the front, until the spots are fully defined.

Discussion

Ultraviolet Lamps.—Six of the eleven organophosphate pesticides, namely, II, III, IV, VI, VII, XI, are located in the chromatograms by the quenched areas visible in the short wave ultraviolet light. They are identified, singly or from a mixture of all eleven, by their order of migration (R_F values). Although the actual R_F values may differ somewhat from chromatogram to chromatogram, or when different quantities are chromatographed, the pattern remains the same for any given solvent system.

For the nonaqueous system, DMF/2,2,4-trimethylpentane, the ascending order is IV, VI, III, VII, XI, II; for the aqueous system, the ascending order is XI, II, III, VII, VI, IV. All except II can be detected at the microgram level; II requires a concentration of 2 mmg or more, depending on the distance the substance migrates. On exposure to the germicidal light, as in blueprinting, IV changes from a quenched area to a fluorescent area. The areas visible in ultraviolet light may be reproduced for inspection in daylight by "printing" onto blueprint paper with the aid of a germicidal lamp (1, 2).

Chromogenic Agents.—The organophosphate pesticides may be located in the chromatograms:

(*a*) By viewing under the short wave ultraviolet light, which locates II, III, IV, VI, VII, XI.

(*b*) By blueprinting, which shows II, III, IV, VI, VII, XI. Exposure to the germicidal light changes IV from a quenched area to a fluorescent area.

(*c*) By exposure to bromine fumes followed by spraying with a neutral solution of fluorescein (a modification of Cook's chromogenic agent (3, 4) for Systox and other organophosphate pesticides). This agent locates all eleven compounds.

(*d*) By spraying with ammoniacal silver nitrate followed by exposure to germicidal light. This agent, as well as (*e*) below, locates all of the compounds except VIII. As a rule 5 mmg quantities of the compounds, I, II, (the nonquenching component), III, IV, V, VI, VII, XI, appear as white areas, IX as a dark or black area against a rather dark brown background, and X (2 components) with little contrast against the background.

(*e*) By spraying after blueprinting, with 2-phenoxyethanol-silver nitrate followed by heating at 130–135°C for 0.5 hour. Five mmg quantities of all compounds, except VIII, show as dark areas against a light brown background.

The chromogenic agents give the most information about the identity of the compounds when papers are developed in duplicate in both the aqueous and nonaqueous solvent systems. All chromatograms are viewed, blueprinted, and viewed again. Then one duplicate is treated with bromine-fluorescein and the other with ammoniacal silver nitrate-germicidal light.

Standard Solutions.—In general, Solutions B were used for chromatographing the single or individual compounds (except that Solution A was used for VIII) and Solutions C were used for the mixture (all eleven compounds); hence 0.001 ml aliquots contain 5, 10, and 2 mmg, respectively, of the compounds. However, 1 mmg quantities of compounds I, III, IV, V, VI, VII, XI were readily detected with the combination of

either the short wave light and bromine-fluorescein, or the short wave light and the silver nitrate chromogenic agent. One component of compound II, the portion which quenches, was detected in chromatograms developed in the aqueous solvent system, probably because of its short migration. One mmg of II was easily detected with the silver nitrate chromogenic agent.

Solvent Systems.—A preliminary run (5) showed that resolution of the eleven compounds under study should be sought among the numerous immobile-mobile aqueous and nonaqueous solvent systems available. Of the aqueous mobile solvents tried (acetic acid, acetone, acetonitrile, the alcohols C_1–C_4, dimethylformamide, dioxane, 2-methoxyethanol, pyridine) with 5, 10, 15, 20% USP Heavy Mineral Oil as the immobile solvent, the system with 10% oil and 50% DMF, v/v, gave the best resolution. Of the nonaqueous immobile solvents tried (various percentages of acetic anhydride, dimethylcyanamide, dimethylformamide, formamide, 2-phenoxyethanol) with the mobile solvents (*n*-hexane, isohexane, *n*-heptane, mixed octanes, 2,2,4-trimethylpentane), the system 20% DMF (v/v) and 2,2,4-trimethylpentane gave the best resolution.

The procedure for the aqueous system directs that the papers be dipped into the immobile solvent before the mobile solvent is transferred to the troughs. This order is reversed for the nonaqueous system for this reason: in the aqueous system, the oil of the immobile solvent is nonvolatile but the mobile solvent is somewhat volatile, and the two components may change in composition if the solvent is allowed to stand in the troughs for variable periods. On the other hand, the one-component mobile solvent of the nonaqueous system does not change in composition, but the DMF in the immobile solvent is more or less volatile and the composition may change. All steps, of course, should be completed in the fastest time possible.

Presentation of Data

Tables of R_F Values.—Table 1, which serves as an index to the four succeeding tables, lists the sample numbers, the common or trademark names, and the chemical names. The samples are arranged alphabetically by their common or trademark names and identified by roman numerals. The chemical names are those used by Metcalf (6).

Table 2 gives the R_F values (the average of 8 observations) for each of the eleven pesticides as resolved by the two solvent systems which contain DMF either as component of the mobile solvent (aqueous system) or as the immobile solvent (nonaqueous system). For the two nonaqueous systems that contain formamide as the immobile sol-

Table 1. Index to Tables 2, 3, 4, and 5

Sample Number	Common or Trade Name	Chemical Name[a]
I	Delnav	2,3-*p*-dioxanedithiol S,S-bis-(O,O-diethylphosphorodithioate)
II	Diazinon	O,O-diethyl O-(2-isopropyl-4-methyl-6-pyrimidyl) phosphorothionate
III	EPN	O-ethyl O-*p*-nitrophenyl phenylphosphorothionate
IV	Guthion	O,O-dimethyl S-(1,2,3-Benzotriazinyl-4-keto)-3-methyl phosphorodithionate
V	Malathion	O,O-dimethyl S-(1,2-dicarboethoxyethyl) phosphorodithionate
VI	Methylparathion	O,O-dimethyl O-*p*-nitrophenyl phosphorothionate
VII	Parathion	O,O-diethyl O-*p*-nitrophenyl phosphorothionate
VIII	Phosdrin	O,O-dimethyl O-1-methoxycarbonyl-1-propen-2-yl phosphate
IX	Ronnel (Trolene)	O,O-dimethyl O-(2,4,5-trichlorophenyl) phosphorothionate
X	Systox (Demeton)	O,O-diethyl O-(and S-)-ethyl-2-thioethyl phosphorothioates
XI	Trithion	O,O-diethyl S-(*p*-chlorophenylthio)-methyl phosphorodithioate

[a] Metcalf, Robert L., *Bull. Entomological Society America*, 5, 5 (1959 March).

Table 2. R_F values (average) for eleven organophosphate pesticides

Quantity of Pesticide in Micrograms (Solids) or Microliters (Liquids)	Temperature, °C	Chromogenic Agent	Order of Intensity of Spots	Organophosphate Pesticide										
				I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Aqueous System — Immobile, 10% Mineral Oil; Mobile, 50% DMF														
5	24	B,F	1	.18-.39	.24	.34	.89	.81	.81	.51	.98	.12	.34	.06
			2		.96								.90	
1	26	B,F	1	.21-.37	—	.34	.87	.82	.81	.53	—	.11	.34	.06
			2		.96								—	
5	24	U,1A,L-O	1	.15-.33	.22	.32	.86	.81	.79	.49	—	.11	.33	.06
		U,2A,H-E	2		—								.89	
5	25	B,F	1	.15-.35	.20	.30	.85	.78	.78	.45	.97	.11	.32	.06
			2		.95								.86	
5	26	U,1A,L-O	1	.18-.41	.21	.35	.88	.81	.83	.52	—	.12	.34	.07
		U,2A,H-E	2		.94								.88	
1	26	B,F	1	.22-.37	.22	.34	.89	.82	.83	.51	—	.12	.34	.07
			2		—								—	
1	26	U,1A,L-O	1	.18-.35	.21	.32	.87	.87	.79	.48	—	.12	—	.05
		U,2A,H-E	2		—								—	
Nonaqueous System — Immobile, 20% DMF; Mobile, 2,2,4-Trimethylpentane														
5	25	B,F	1	.80	.48	.69	.10	.62	.35	.75	.11	.96	.96	.96
			2	.95	.98						.04		.59	
2	25	B,F	1	.80	.48	.70	.10	.64	.36	.76	.11	.94	.94	.94
			2	.96	.97						.04		.57	
1	26	B,F	1	.71	.45	.59	.07	.48	.29	.66	—	.93	.93	.92
			2		—						—		—	
5	24	1A,L	1	.75	—	.61	.02	.55	.30	.69	.10	.83	.93	.97
			2		.97						.03		.56	
5	26	1A,L-O	1	.55	—	.43	.04	.43	.21	.55	—	.89	.91	.85
		2A,H-E	2		.97						—		.50	
1	26	B,F	1	.62	.40	.50	.07	.49	.26	.60	.11	.90	.91	.88
			2								.06		.47	
1	26	1A,L-O	1	.67	—	.54	.09	.49	.26	.62	—	.93	—	.93
		2A,H-E	2								—		—	
Nonaqueous System — Immobile, 10% Formamide; Mobile, 2,2,4-Trimethylpentane														
5	24	B,F	1				.51				.15			
			2								.04			
Nonaqueous System — Immobile, 30% Formamide; Mobile, 2,2,4-Trimethylpentane														
5	24	B,F	1				.30	.90	.72		.07		.62	
			2								.01		—	

vent, the R_F values are given only for those pesticides which are resolved from the other ten under study. It should be noted, however, that only the lower component of X is resolved from all of the pesticides. Table 2 also shows the quantities of the pesticides chromatographed (except VIII, which is always 10 mmg when spotted singly); the room temperature during development; the chromogenic agent used; and the order of intensity of the spots if the solvent system resolves the two isomers that pesticides I, II, VIII, and X are said to contain (7).

To save space, the chromogenic agents are

coded in the tables to show the agents used, their sequence, and any variation in treatment of the two papers developed in each tank. The code is as follows:

B: The air-dry chromatogram was fumed in bromine vapors for about 30 seconds.

F: The chromatogram was sprayed with the fluorescein solution within about 30 seconds after it was fumed in bromine vapors. The chromatogram was then examined in the short wave light within 3 to 5 minutes of the fluorescein spraying, and the spots were circled with a soft pencil.

U: The chromatogram was exposed to the germicidal light for about 25 minutes, as in blueprinting, or was actually blueprinted.

1A: The exposed paper was sprayed with the ammoniacal silver nitrate agent, (b).

L: The sprayed paper was exposed to the germicidal light, first the back side, then the front, until the spots were fully defined.

O: The odd-numbered sheets were treated in the above sequence.

2A: The exposed paper was sprayed with 2-phenoxyethanol silver nitrate agent, (c).

Table 3. R_F values (average) for the six organophosphate pesticides which quench the short wave ultraviolet light, 2537Å, as resolved from a mixture of all eleven

Quantity of Pesticide in Micrograms (Solids) or Microliters (Liquids)	Temperature, °C	Chromogenic Agent	Order of Intensity of Spots	Organophosphate Pesticide										
				I	II	III	IV	V	VI	VII	VIII	IX	X	XI
Aqueous System — Immobile, 10% Mineral Oil; Mobile, 50% DMF														
5	24	B,F	1		.23	.30	.86		.78	.48				.05
2	24	B,F	1		.24	.33	.87		.78	.48				.07
1	26	B,F	1		—	.33	.87		.78	.49				.06
2	25	B,F	1		.20	.32	.86		.77	.47				.06
2	26	1A,L-O	1		.23	.35	.87		.80	.50				.07
1	26	B,F	1		.21	.34	.87		.79	.50				.07
1	26	1A,L-O	1		.20	.31	.86		.77	.46				.06
Nonaqueous System — Immobile, 20% DMF; Mobile, 2,2,4-Trimethylpentane														
2	25	B,F	1		.97	.68	.10		.35	.74				.94
1	26	B,F	1		—	.60	.08		.29	.68				.92
5	24	1A,L	1		.96	.63	.08		.31	.70				.92
2	26	1A,L-O	1		.96	.45	.05		.21	.56				.85
1	26	B,F	1		—	.50	.06		.25	.59				.88
1	26	1A,L-O	1		—	.57	.08		.28	.65				.93
Nonaqueous System — Immobile, 10% Formamide; Mobile, 2,2,4-Trimethylpentane														
5	24	B,F	1				.49				.15			
			2								.03			
Nonaqueous System — Immobile, 30% Formamide; Mobile, 2,2,4-Trimethylpentane														
5	24	B,F	1				.30		.74		.07			
			2								.01			

Table 4. One-two-two-one-dimensional chromatography. R_F values for each of the eleven organophosphate pesticides

Temperature, °C	Chromogenic Agent	Order of Intensity of Spots	Dimensional Chromatography	Direction of Mobile Solvent	Organophosphate Pesticide										
					I	II	III	IV	V	VI	VII	VIII	IX	X	XI
24	B,F	1	One	↑	.78	.46	.70	.07	.55	.22	.76	.10	.96	.95	.96
			Two	↑	.80	.47	.70	.05	.57	.23	.70	.10	.96	.96	.96
			Two	→	.28	.94	.32	.86	.78	.81	.43	—	.08	.31	.03
			One	→	.26	.93	.30	.85	.74	.77	.43	.95	.08	.31	.04
		2	One	↑		.97 Q						.04		.55	
			Two	↑		.98						.04		.60	
			Two	→		.20						—		.88	
			One	→		.21						.95		.88	
26	1A,L	1	One	↑	.65	—	.55	.05	.50	.24	.64	—	.92	.93	.93
			Two	↑	.69	—	.55	.07	.50	.26	.64	—	.94	.95	.93
			Two	→	.30	—	.35	.89	.82	.84	.53	—	.11	.30	.07
			One	→	.29	—	.30	.87	.78	.81	.48	—	.09	.31	.06
		2	One	↑		.97 Q								.51	
			Two	↑		.97								.59	
			Two	→		.20								.87	
			One	→		.21								.90	
25	B,F	1	One	↑	.86	.47	.89	.12	.67	.36	.80	.08	.97	.97	.99
			Two	↑	.90	.51	.89	.11	.65	.40	.79	.02–.08	.97	.98	.99
			Two	→	.22	.96	.33	.89	.77	.79	.47	.96	.08	.27	.03
			One	→	.21	.99	.30	.85	.76	.77	.44	.95	.08	.30	.04
		2	One	↑		.97 Q						.02		.59	
			Two	↑		.99						.02–.08		.59	
			Two	→		.17						.96		.94	
			One	→		.21						.95		.89	

H: The sprayed paper was heated at 130–135°C for 30 minutes.

E: The even-numbered sheets were treated in the above sequence.

The dash (—) in the tables indicates no spot, or one so weak that it has little, if any significance. It may also mean that a spot appears in one chromatogram but not in another, although the same amount of the pesticide was chromatographed, because of the non-contrasting background due to the presence of greater or less quantities of impurities that were not removed when the paper was prewashed with water. A band of impurities may concentrate in the paper during development, next to or near the aqueous (but not the nonaqueous) solvent front, and may react with the chromogenic agent. This band is called the "curtain"; it contains both silver and bromine reactants.

The notation, .18–.39, signifies a spot so long that it is essentially a streak. This streak appears for I in the aqueous system only and may represent a partial separation of the alpha-beta isomers. The R_F values represent spots visible in daylight or in the short wave light.

Table 3 lists the R_F values (the average of 16 observations) for those pesticides which were resolved from mixtures of all 11 organophosphate pesticides and definitely identified as the individual pesticide. The pesticides for which no R_F values are shown could not be positively identified in this manner. The dash (—) under II shows that it was not detected at the 1 mmg level.

Table 4 shows the R_F values obtained in 3 experiments for 5 mmg quantities of each pesticide resolved simultaneously by one and two-dimensional chromatography on the same sheet of filter paper. The most striking feature is the wide variation in the R_F values

among some of the pesticides and the narrow spread for the others. The pattern of resolution, however, remains the same for each pesticide.

Table 5 gives the R_F values (the average of 5 observations) for 8 or 9 of the 11 pesticides (depending on the chromogenic agent) resolved by two-dimensional chromatography from a mixture which contains 5 mmg of each compound. The six pesticides, II, III, IV, VI, VII, XI, which show quenched areas in ultraviolet light are identified by one-dimensional chromatography of a mixture that contains 2 mmg each of the 11 compounds. The ammoniacal silver nitrate chromogenic agent yields a dark area for IX because of its chlorine content, and thus distinguishes it from the others.

Figures.—Figures 1 to 16 were selected to illustrate the various phases of resolution and identification of the eleven organophosphate pesticides. It will be noted that all, except X, can be separated from each other and identified in any combination of the pesticides.

In view of the limited space, the pesticides are identified in the figures by arabic numbers instead of the Roman numerals used throughout the report.

Figure 1 illustrates the resolution of II, III, IV, VI, VII, XI, singly and from a mixture of all eleven pesticides. The quenched areas in the chromatogram were first located (dotted) under the short wave ultraviolet light and then circled under the light after the chromatogram was fumed with bromine and sprayed with fluorescein.

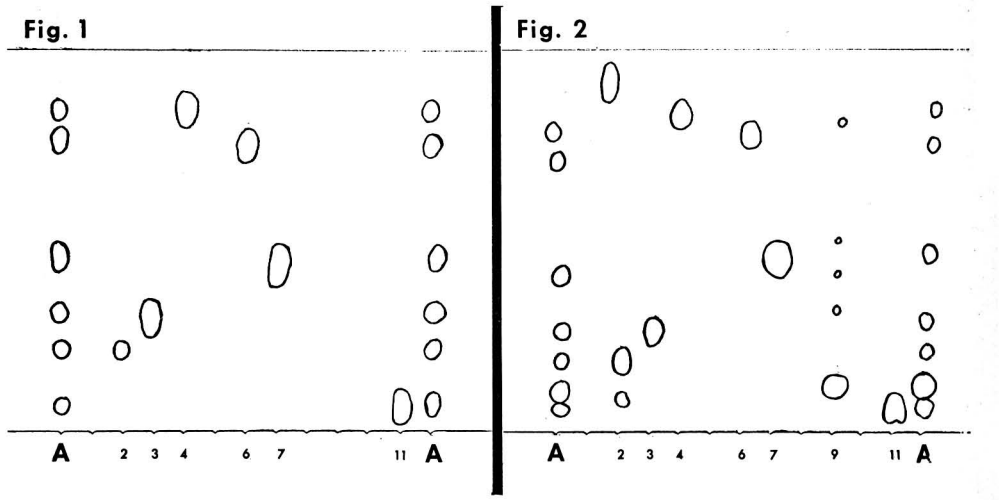
Figure 2 repeats 1, except that the quenched areas were circled after the chromatogram was sprayed with ammoniacal silver nitrate and exposed to the germicidal light. The six compounds in Figure 1 above appear as white areas against a brown background. In addition, IX appears as a dark or black area. Further, II yields two other compounds and IX shows four impurities, singly but not from the mixture.

Figure 3 repeats 1, except that the nonaqueous solvent system, DMF-2,2,4-trimethylpentane, was used. The two solvent systems, aqueous and nonaqueous, essentially reverse the order of migration of the six pesticides.

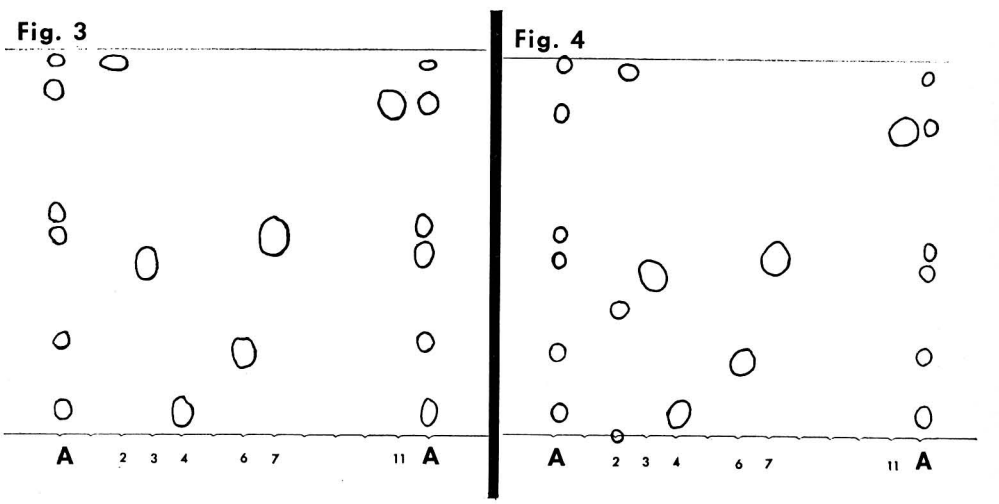
Figure 4 repeats the solvent system of 3. The areas in the chromatogram were circled after it was sprayed with 2-phenoxyethanol-silver nitrate and heated at 130°C for 30 minutes. This chromogenic agent yields dark or black areas against a brown background; the quenched area for II is barely discernible at the 5 mmg level, probably because it migrates the length of the paper just below

Table 5. Two-dimensional chromatography. R_F values (average) for eight organophosphate pesticides resolved from a mixture of all eleven with one-dimensional R_F values for the six pesticides which quench the short wave ultraviolet light

Temperature, °C	Chromogenic Agent		Dimensional Chromatography	Direction of Mobile Solvent	Organophosphate Pesticide										
					I	II	III	IV	V	VI	VII	VIII	IX	X	XI
24	B,F		One	↑		.98	.65	.07		.31	.73				.94
			Two	↑	.78	.98	.65	.08	.60	.30	.72				.95
			Two	→	.25	.18	.29	.83	.74	.74	.44				.04
			One	→		.18	.25	.81		.71	.40				.03
26	U,1A,L		One	↑		.98	.52	.05		.25	.61				.90
			Two	↑	.65	.99	.53	.05	.46	.22	.57		.94		.93
			Two	→	.24	.15	.30	.84	.78	.78	.46		.08		.04
			One	→		.15	.27	.83		.75	.42				.04
26	U,B,F		One	↑		—	.72	.11		.37	.78				.97
			Two	↑	.85	.99	.73	.12	.64	.38	.79				.98
			Two	→	.24	.15	.25	.87	.77	.77	.45				.04
			One	→		.16	.25	.83		.75	.42				.03



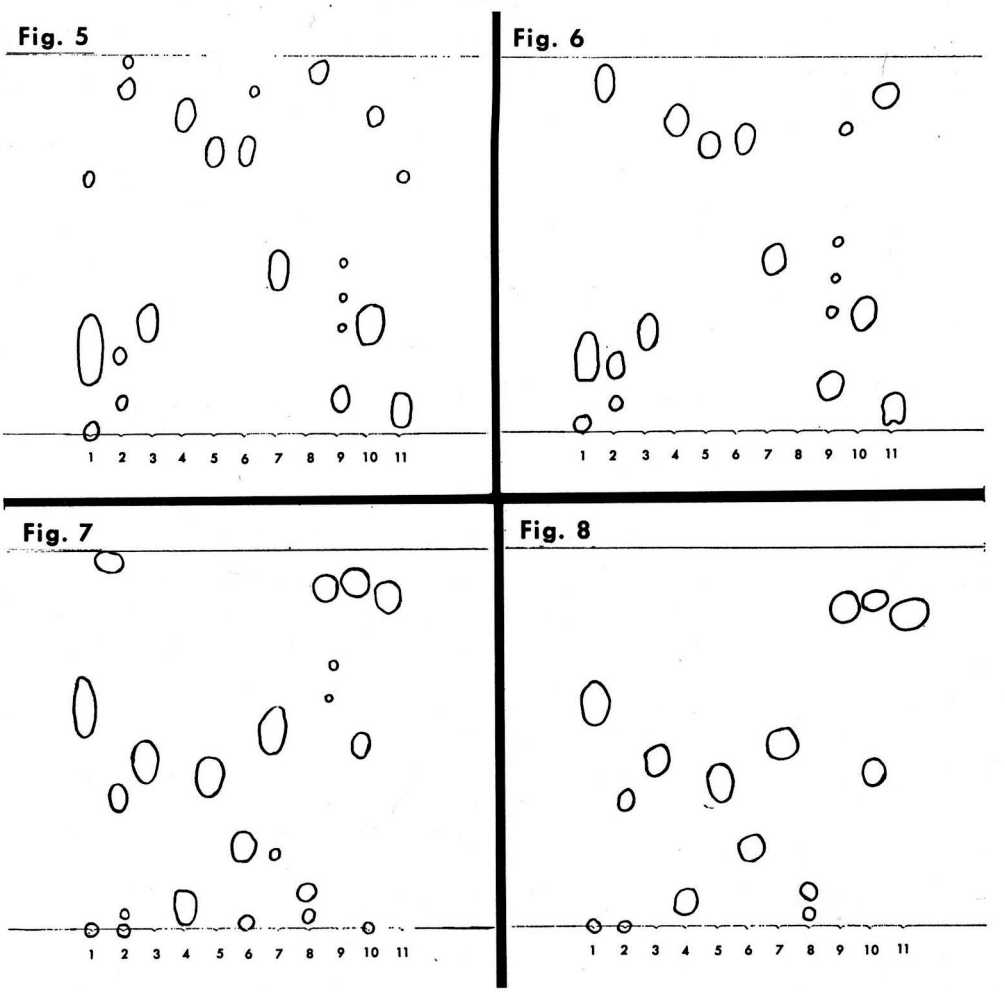
Figs. 1 and 2—Migration properties of II, III, IV, VI, VII, XI (both figures) and IX (Fig. 2); ascending chromatography. Aqueous solvent system: immobile solvent, 10% (v/v) heavy mineral oil in ethyl ether; mobile solvent, 50% (v/v) DMF in water. Chromogenic agents, short wave (2537 Å) ultraviolet light followed by: Fig. 1, bromine and fluorescein; Fig. 2, ammoniacal silver nitrate and germicidal light. Five mmg of each pesticide was applied singly at serrations 1–7 and 9–11, 10 mmg at 8, and 2 mmg each of all 11 pesticides at the two serrations A. Only the six pesticides that show quenched areas in the chromatogram (Fig. 1) and these six plus the one which shows a dark (black) area in the chromatogram (Fig. 2) were circled. Separates II, III, IV, VI, VII, XI (Fig. 1), and II, III, IV, VI, VII, IX, XI (Fig. 2) from any combination of the pesticides. Compare Fig. 1 and Fig. 5; Fig. 2 and Fig. 6.



Figs. 3 and 4—Migration properties of II, III, IV, VI, VII, XI; ascending chromatography. Nonaqueous solvent system; immobile solvent, 20% (v/v) DMF in ethyl ether; mobile solvent, 2,2,4-trimethylpentane. Chromogenic agents, short wave (2537 Å) ultraviolet light followed by: Fig. 3, bromine and fluorescein; Fig. 4, 2-phenoxyethanol-silver nitrate and heat at 130° C. Five mmg of each pesticide was applied singly at serrations 1–7, and 9–11, 10 mmg at 8, and 2 mmg each of all 11 pesticides at the two serrations A. Only the six pesticides which show quenched areas in the chromatogram were circled. Separates II, III, IV, VI, VII, XI from any combination of the pesticides. Compare Fig. 3 and Fig. 7; Fig. 4 and Fig. 8.

the solvent front. Another much darker area moved about one-third of the distance that the front traveled, and there is also a residual area; yet the compound is labeled at 99.2% pure.

Figures 5, 6, 7, and 8 are repeats of 1,



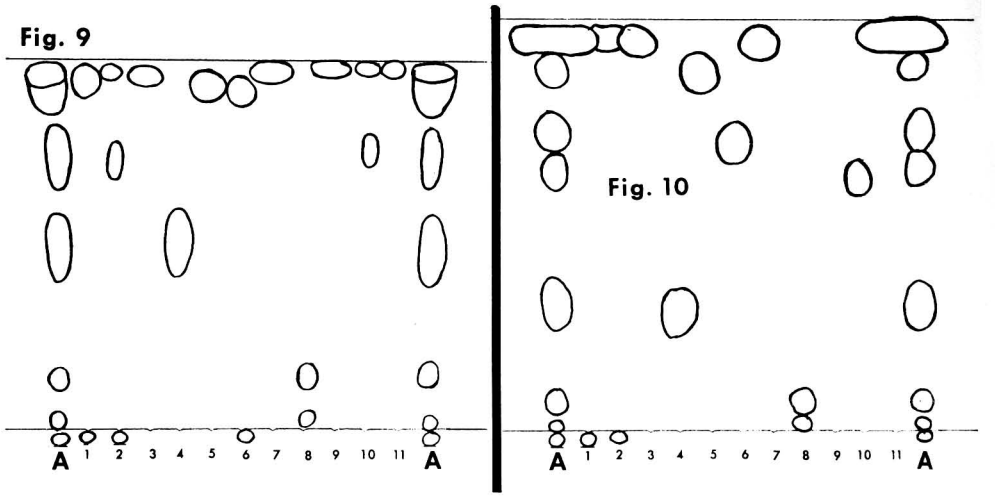
Figs. 5, 6, 7, 8—Migration properties of the 11 pesticides; ascending chromatography. **Figs. 5 and 6, aqueous solvent system: immobile solvent, 10% (v/v) heavy mineral oil in ethyl ether; mobile, 50% (v/v) DMF in water. Figs. 7 and 8, nonaqueous solvent system: immobile solvent, 20% (v/v) DMF in ethyl ether; mobile solvent, 2,2,4-trimethylpentane. Chromogenic agents: Figs. 5 and 7, bromine and fluorescein; Fig. 6, short wave (2537 Å) ultraviolet light followed by ammoniacal silver nitrate and germicidal light; Fig. 8, 2-phenoxyethanol-silver nitrate and heat at 130° C. Five mmg of each pesticide was applied at serrations 1–7 and 9–11, and 10 mmg at 8. Compare Figs. 5 and 1; Figs. 2 and 6; Figs. 3 and 7; Figs. 4 and 8.**

2, 3, and 4, respectively, using all eleven pesticides instead of the six which show quenched areas under the short wave ultraviolet light.

Figure 9, which illustrates the resolution of all eleven pesticides, singly and in mixture, shows the separation and identification of IV and the two isomers of VIII from all of the compounds, and the separation of the nonfluorescent component of II and the lower isomer of X from the other pesticides, but not from each other.

Figure 10 shows the separation and identification of IV, V, VI, the two isomers of VIII, and the lower isomer of X from all other pesticides.

Figure 11 illustrates the pattern of migration for II, III, IV, VI, VII, XI, the pesticides which show quenched areas under the short wave ultraviolet light, when they are resolved simultaneously in the same sheet of filter paper by two-dimensional chromatography and identified by one-dimensional chromatography. The chromogenic agent



Figs. 9 and 10—Migration properties of all 11 pesticides, singly and mixtures of all 11; ascending chromatography. Nonaqueous solvent system: immobile solvent, Fig. 9, 10% (v/v) formamide in ethyl ether; Fig. 10, 30% (v/v) formamide in ethyl ether; mobile solvent, 2,2,4-trimethylpentane. Chromogenic agent, bromine and fluorescein. Five mmg of each pesticide was applied respectively at serrations 1–11, and mixture at the two A's. Fig. 9 shows the separation of IV and the two isomers of VIII from any combination of the pesticides. Fig. 10 shows the separation of IV, V, VI, the two isomers of VIII, and the lower isomer of X from any combination of the pesticides.

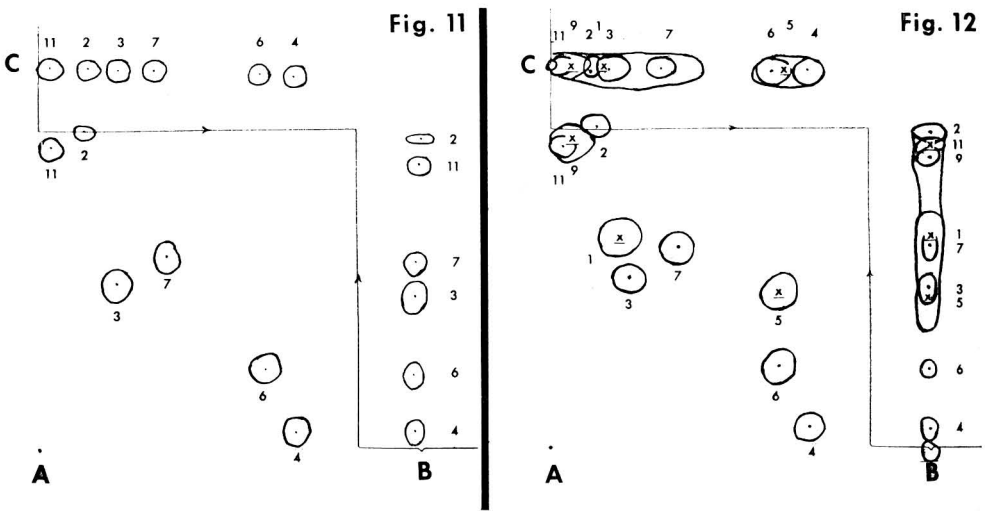
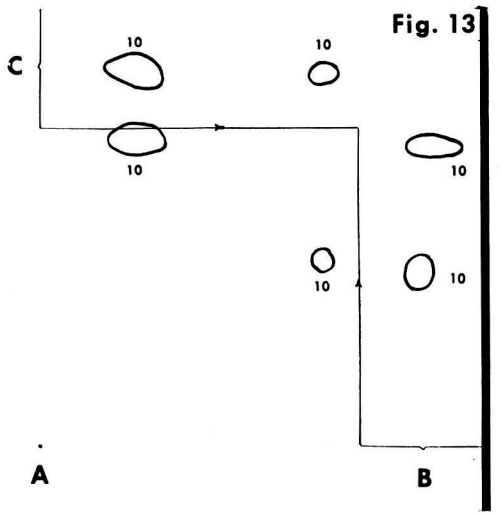
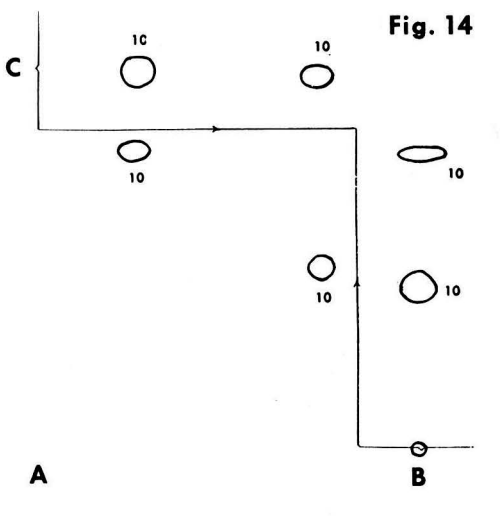


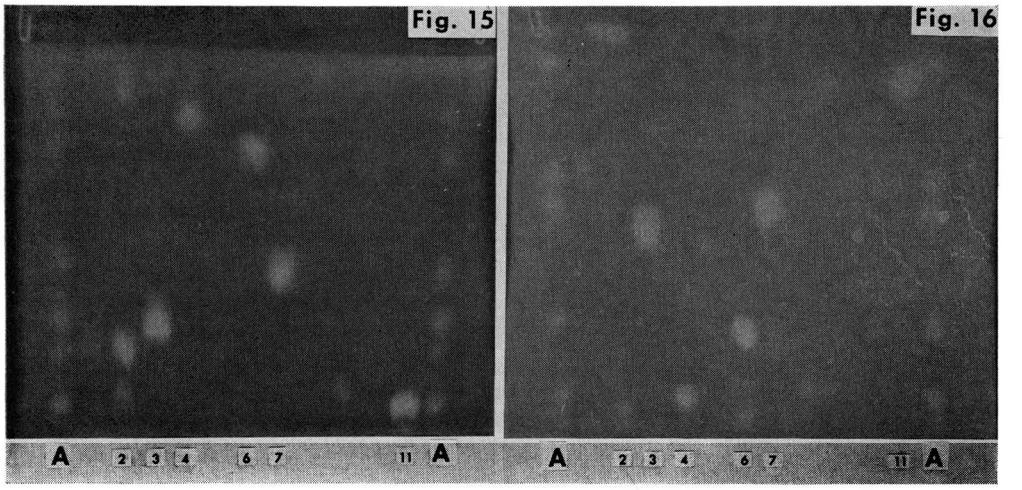
Fig. 11—Migration pattern of II, III, IV, VI, VII, XI; two-dimensional chromatography. Chromogenic agent, short wave (2537 Å) ultraviolet light followed by ammoniacal silver nitrate and germicidal light. Identification, one-dimensional. Five mmg of each pesticide (all 11) was applied at dot A and 2 mmg of each pesticide (all 11) at serrations B, perpendicular, and C, horizontal. Order of migration: perpendicular, IV, VI, III, VII, XI, II; horizontal, XI, II, III, VII, VI, IV. Separates II, III, IV, VI, VII, XI from any combination of the pesticides.

Fig. 12—Migration pattern of I, II, III, IV, V, VI, VII, IX XI; two-dimensional chromatography. Chromogenic agent, ammoniacal silver nitrate and germicidal light. Identification, one-dimensional. Five mmg of each pesticide (all 11) was applied at dot A and 2 mmg of each pesticide (all 11) at serrations B, perpendicular, and C, horizontal. Order of migration: perpendicular, IV, VI, V, III, VII, I, XI, IX, II; horizontal, XI, IX, II, I, III, VII, VI, V, IV. Separates I, II, III, IV, V, VI, VII, IX, XI from any combination of the pesticides. • = quenched areas visible under short wave ultraviolet light. × = not visible under ultraviolet light until after treatment with ammoniacal silver nitrate plus germicidal light.





Figs. 13 and 14—Migration properties of the two isomers of X; two-dimensional chromatography. Chromogenic agents: Fig. 13, bromine and fluorescein; Fig. 14, ammoniacal silver nitrate and germicidal light. Identification, one-dimensional. Five mmg of X was applied at dot A and serrations B, perpendicular, and C, horizontal.



Figs. 15 and 16—Blueprints of the separation of II, III, IV, VI, VII, XI from any combination of the pesticides; ascending chromatography. Fig. 15, aqueous solvent system; immobile solvent, 10% (v/v) heavy mineral oil in ethyl ether; mobile solvent, 50% (v/v) DMF in water. Fig. 16, nonaqueous solvent system: immobile solvent, 20% (v/v) DMF in ethyl ether; mobile solvent, 2,2,4-trimethylpentane. Five mmg of each pesticide was applied singly at serrations 1–7 and 9–11, 10 mmg at 8, and 2 mmg each of all 11 pesticides at the two serrations A. Compare the two figures for order of migration.

may be either bromine-fluorescein or ammoniacal silver nitrate-germicidal light. The order of resolution for the first run, perpendicular, is IV, VI, III, VII, XI, II, and for the second run, horizontal, is IX, II, III, VII, VI, IV.

Figure 12 illustrates the pattern of migration for I, II, III, IV, V, VI, VII, IX, XI, when they are resolved simultaneously in the same sheet of filter paper by two-dimensional chromatography and identified by one-dimensional chromatography. Ammoniacal silver nitrate-germicidal light was used as chromogenic agent because IX gives a dark or black spot that can be distinguished from II and XI which migrate to the same portion

of the chromatogram. The order of resolution for the first run, perpendicular, is IV, VI, V, III, VII, I, XI, IX, II, and for the second run, horizontal, is XI, IX II, I, III, VII, VI, V, IV. The pesticides are more readily identified and distinguished from one another if the quenched areas are dotted with a soft pencil before the chromatogram is treated with the chromogenic agent and if 2 mmg quantities of each pesticide are used for one-dimensional work and 5 mmg for two-dimensional chromatography.

Figures 13 and 14 illustrate the migration properties of the two isomers of pesticide X. The chromatogram treated with the bromine-fluorescein chromogenic agent shows no residual spot, 13, but the one treated with ammoniacal silver nitrate-germicidal light shows a spot, 14. Note that the order of migration of the isomers differs with the two systems. No paper chromatographic procedure was found adequate to separate and identify X in a mixture of the eleven organophosphate pesticides covered in this study.

Figures 15 and 16 are photographs of blueprints. They illustrate not only the migration properties but also the separation and identification of II, III, IV, VI, VII, and XI from all of the pesticides. They are located in the chromatograms by their quenched areas under the short wave ultraviolet light rather than by chromogenic agents, and made visible in daylight by blueprinting. Note the two orders of separation by the aqueous and nonaqueous solvent systems.

Summary

Paper chromatographic techniques are described for the separation and identification of the following organophosphate pesticides: (I) Delnav, (II) Diazinon, (III) EPN, (IV) Guthion, (V) malathion, (VI) methyl parathion, (VII) parathion, (VIII) Phosdrin, (IX) Ronnel, (X) Systox, and (XI) Trithion. All, except Systox, can be separated from any combination of the pesticides. Certain separations are made with one-dimensional chromatography, others with two-dimensional. Four solvent systems are used, one aqueous and three nonaqueous. The aqueous solvent consists of 10%, v/v, heavy mineral oil in ethyl ether as the immobile solvent and 50%, v/v, DMF in water as the mobile solvent. The nonaqueous system consists of 20% DMF, v/v, in ethyl ether, or 10% or 30% formamide, v/v, in acetone as the immobile solvent, and 2,2,4-trimethylformamide as the mobile solvent. The compounds are located in the chromatogram by a system of five chromogenic agents.

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A Quantitative Method for the Determination of 2,3-Dichloro-1,4-Naphthoquinone (Phygon) Residues in Cherry Extracts

By W. P. McKINLEY and SHIRLEAN A. MAGARVEY (Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Ontario, Canada)

The fungicide Phygon, 2,3-dichloro-1,4-naphthoquinone, has been used as a fungicide on a number of edible crops, on seeds, and as an algacide in water (1).

Burchfield and McNew (2) described an analytical method for Phygon in acetone extracts of seeds which depends on a reaction between Phygon and dimethylamine in aqueous acetone. The red reaction product had an absorbance maximum at 495 mμ. This method was then modified by Lane (3) for the analyses of Phygon on peaches, apples, strawberries, string beans, and tomatoes. Lane used benzene as the extracting solvent in order to remove less colored plant material, and employed larger samples and 10 cm cells, instead of the 1 cm cells, to increase the sensitivity. Bornmann (4) used essentially the same method for the analyses of extracts of celery and tomatoes. Newell, Mazaika, and Cook (1) measured micro amounts of Phygon in water by ultraviolet absorption. Sodium chloride was added to the water, which was acidified with phosphoric acid. The Phygon was then removed by steam distillation.

In this laboratory, it was found that the highly volatile dimethylamine was an undesirable material to handle. The anhydrous liquid was difficult to measure, even when precooled, and some of the reagent was lost from closed cells. A reagent prepared from triethylamine was used in the work presented here.

METHOD

Reagents

(a) *2,3-Dichloro-1,4-naphthoquinone.*—M.p. 195–196°C (Eastman Organic Chemicals 3836), or technical Phygon purified as described by Lane (3).

(b) *Dimethylamine (anhydrous).*—Eastman Organic Chemicals 601.

(c) *Diethylamine.*—Eastman Organic Chemicals 92.

(d) *Triethylamine.* — Eastman Organic Chemicals 616 or BDH reagent grade (prepared as described in the text).

(e) *Florisil.*—MgO (15%), Na_2SO_4 (½%), and SiO_2, 60–100 mesh (Floridin Co., Tallahassee, Fla.).

(f) *Ethanol.*—Absolute. (Gooderham and Worts, Ltd.).

(g) *p-Toluene sulfonyl chloride.*—Eastman Organic Chemicals 523, purified by distilling under reduced pressure.

Apparatus

(a) *Ultraviolet light.*—Hanovia Arc, 5 amp. lamp (Hanovia Chemical Co., Newark, N.J.).

(b) *Cooling bath.*—Water containing 50% ethylene glycol used as the cooling liquid. The temperature was held at 0°C by means of a refrigerator unit (5).

Preparation of Triethylamine Reagent and Standard Curve

(a) Place 300 ml triethylamine in a 500 ml glass-stoppered reagent bottle. Place bottle on its side in a wire basket, in such a manner that the triethylamine is below the surface of the cooling liquid, while the remainder of the bottle, including the stopper, is above the surface. Place ultraviolet lamp with the filter removed on the bath approximately 3 inches above the bottle and expose liquid to the light for approximately 48 hours. Add 100 mg *p*-toluene sulfonyl chloride to the reagent and stir mixture for 15 minutes with a magnetic stirrer. Place mixture in the freezing compartment of a refrigerator for one hour and filter through a Whatman No. 42 filter paper. Store filtered reagent in a brown bottle in the refrigerator overnight, and refilter. (The reagent is nearly colorless.) Let 2.5 ml of the reagent, 0.5 ml of the Phygon standard (50 mmg), and 1.5 ml of absolute ethanol react for 10 minutes at 25°C, and measure against a reagent blank at 672 mμ. Absorbance reading should be 0.55 ± 0.08.

If too little *p*-toluene sulfonyl chloride is used, the reaction product is purple. Too great a quantity of *p*-toluene sulfonyl chloride, however, causes the reagent to become yellow and

may yield a greenish-blue product rather than the desired blue.

(*b*) Dissolve a 50.0 mg portion of Phygon in absolute ethanol and dilute to 500 ml (100 mmg per ml) for the stock solution. Make 25 ml of this stock solution to 100 ml with ethanol and label as standard solution (25 mmg per ml). Measure aliquots of 0.0, 0.5, 1.0, 1.5, 2.0, and 2.5 ml of the standard solution into 10 ml glass-stoppered cylinders and add sufficient absolute ethanol to each cylinder to make a volume of 2.5 ml. Add 2.5 ml of triethylamine reagent to each cylinder. Shake cylinders and place in a constant temperature bath at 25°C for 10 minutes. Read absorbance of the standards and reagent blank in 1 cm cells in a Beckman Model B spectrophotometer, or equivalent instrument, at 672 mμ.

Preparation of Sample

Prepare samples by the method of Gunther and Blinn (6), then weigh 900–1100 g of each sample to the nearest 5 g and place in a one gallon jar. Strip samples with 500 ml acetone in the manner described earlier (7), except strip for 10 minutes (4).

Place acetone extracts, in 600 ml beakers, in a water bath at 60°C under a gentle stream of air until all acetone is removed. Transfer water remaining to a separatory funnel, and rinse beaker with three 20 ml portions carbon tetrachloride and add rinsings to the separatory funnel. Shake mixture and remove carbon tetrachloride. Extract aqueous phase two more times, using 20 ml carbon tetrachloride for each extraction. Place combined extracts in the bath at 60°C and remove solvent. Discard aqueous phase, which contains most of the pigments. Dissolve residue in 20 ml benzene, and add this to a Florisil (60/100 mesh) column (15 × 300 mm). Rinse beaker with two more 20 ml volumes benzene and add to the column. Allow extract to enter the column and add an additional 150 ml benzene and collect eluate in a 250 ml beaker. Place beakers in the steam bath and remove solvent under a gentle stream of air. Dissolve residue in absolute ethanol and make to 25 ml. Mix a 2.5 ml portion of this ethanolic solution and the same quantity of triethylamine reagent, and allow to react for ten minutes at 25°C. Read resulting colored product at 672 mμ in the manner described for the standard. A 2.5 ml aliquot represents the extract from 100 g of plant material. If this quantity of plant material is used, measure Phygon within the range 0.1 to 0.7 ppm. For larger quantities of Phygon, dilute solution with absolute ethanol before taking the aliquot for analyses.

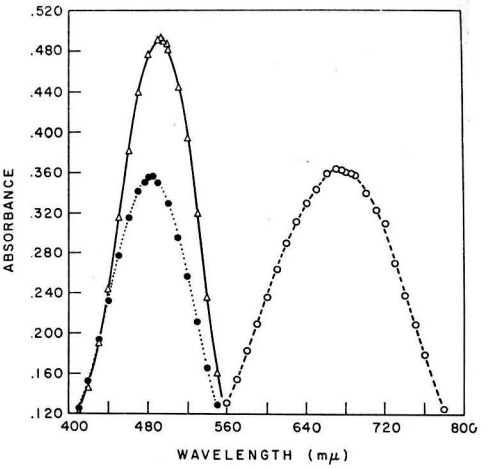

Fig. 1—Absorbance curves for the reaction products of Phygon and each of the following reagents: (1) Solid circles, dimethylamine; (2) Triangles, diethylamine; (3) Hollow circles, triethylamine (UV treated).

Experimental

Absorbance Maxima

An ethanol solution of pure Phygon was prepared to contain 100 mmg per ml solution, and 0.5 ml of solution was placed in each of three stoppered, graduated cylinders. To each cylinder was added 2 ml ethanol, followed by 2.5 ml dimethylamine to the first cylinder, 2.5 ml diethylamine to the second, and 2.5 ml triethylamine reagent (UV treated) to the third. Three reagent blanks, each containing 2.5 ml ethanol and 2.5 ml of the respective amines, were prepared and the absorbance curve for each reaction product was measured against its reagent blank (Fig. 1). The absorbance peaks were 484, 492, and 672 mμ, respectively, for dimethylamine, diethylamine, and triethylamine reagents. The reaction products with the dimethylamine and the diethylamine were orange at low concentrations and red at high concentrations of Phygon. The diethylamine has a much higher boiling point and should be a more desirable reagent than the dimethylamine. The triethylamine reagent has a higher boiling point and its reaction product is a distinct blue, with an absorbance maximum at a wavelength

less susceptible to interference from extraneous material.

Concentration of Reagent

Standards were prepared to contain the same quantity of Phygon and were made to the same volume with absolute ethanol, with varying amounts of reagent from zero to four ml (Fig. 2). Two ml of reagent was sufficient to produce a maximum amount of color and the addition of more than 3 ml reagent produced a slight decrease in color. The addition of 2.5 ml has therefore been recommended in the method.

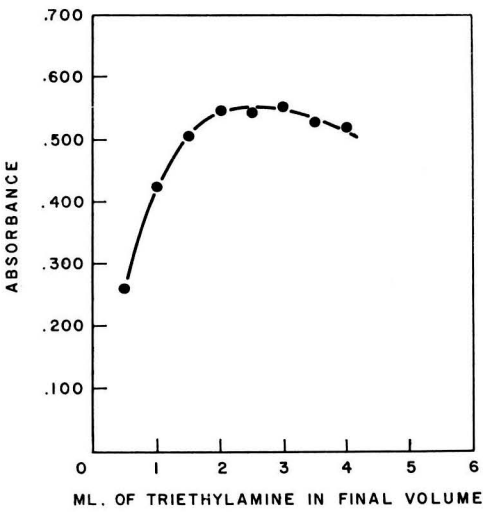

Fig. 2—Absorbance values for the reaction product of Phygon and different amounts of the triethylamine reagent in absolute ethanol at 672 mμ.

Effect of Temperature

Twelve aliquots of the standard, each containing the same amounts of Phygon, ethanol, and triethylamine reagent were prepared with four corresponding reagent blanks. Three cylinders and a reagent blank were allowed to react at each of four temperatures shown in Fig. 3 (20, 25, 30, and 35°C). The solutions were brought to room temperature and read against their reagent blanks. Apparently slightly less color was formed at 30 and 35°C than at the two lower temperatures. The reagent is destroyed when brought to its boiling temperature, indicating that the increase in temperature may affect the reagent rather than the reaction product.

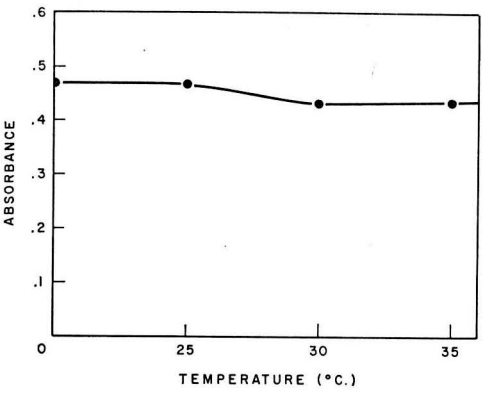

Fig. 3—Absorbance values for the reaction product of Phygon and the triethylamine reagent at four temperatures at 672 mμ.

Time of Reaction and Stability of Product

Three standards and a reagent blank were prepared and the absorbances recorded each minute up to 10 minutes, every 2.5 minutes thereafter up to 20 minutes, and at 25, 30, 45, and 60 minutes. The color developed had reached a maximum at 5 minutes and was stable for at least 1 hour (Fig. 4). Visually the color is stable for several days, whereas readings with the dimethylamine reagent must be made within one hour because the solution fades (4). A stable reaction product is often useful in confirming the identity of pesticide residues in the presence of other substances.

Results and Discussion

Table 1 illustrates the relationship between absorbance and the quantity of Phygon in

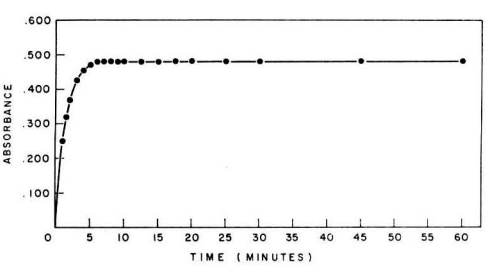

Fig. 4—Absorbance values for the reaction product of Phygon and the triethylamine reagent for different times of reaction at 672 mμ.

Table 1. Relationship between absorbance and amount of Phygon

Phygon (Mmg)	No. of Determinations	Absorbance Range	Absorbance Average	Standard Deviation
12.5	6	0.125–0.145	0.135	0.007
25.0	6	0.270–0.304	0.289	0.011
37.5	6	0.430–0.466	0.448	0.015
50.0	6	0.590–0.625	0.604	0.013
62.5	6	0.760–0.790	0.777	0.010
50.0[a]	12	0.500–0.584	0.548	0.021

[a] The 12 values for 50 mmg Phygon were determined with 3 different lots of reagent.

the final volume. The relationship is linear within the range shown in the table. Although the slope of the line varied slightly with each new batch of reagent, it was always linear and passed through the origin when plotted. The standard deviation for the 12 determinations at the 500 mmg level is slightly larger than for the 6 at the various levels (Table 1), possibly because 3 different lots of reagent were used.

In order to determine the recovery values from the Florisil columns, aliquots of the standard solution of Phygon in benzene were put on the columns and eluted in the manner described for extracts. The eluates were concentrated and made to volume, and the Phygon content was determined. Recoveries were satisfactory at both the 200 and 500 mmg levels, as follows: for 200 mmg, the range was 84.3–101.8%, average 94.7%; for 500 mmg, the range was 83.0–98.5%, average 90.8%.

Recovery values from cherry extracts were obtained in order to determine how efficiently the extraneous material was removed, and whether losses of the Phygon took place in the process. Fresh whole cherries were extracted on a roller stripper with acetone, and the Phygon was added before the solvent was removed. Recovery values for four determinations ranged from 85.6 to 98.7% and averaged 92.5%.

Crop blanks were prepared by extracting 1000 g of Phygon-free cherries. The average blank absorbance reading for eluates (1/10 the total eluate) containing the extract from 100 g of cherries was 0.002, which indicated that extraneous interfering material was almost completely removed.

All of the pesticides listed in a previous publication (7) were tested with the reagent. None of these compounds reacted to give a colored product when present at several times the concentration level which might be expected to be encountered. The only pesticide which gave a color with the reagent was tetrachloro-*p*-benzoquinone (Spergon), and it is not likely that this compound would be used with Phygon. If the two were encountered together, they could be resolved by paper chromatography (8) and identified by spraying the paper with the reagent described in this paper. If the Phygon content is greater than 1.0 ppm, it may be seen moving off the Florisil column as a yellow band. This is a means of identification.

Acknowledgments

The authors are indebted to Dr. D. G. Chapman and Mrs. Constance Webster for constructive criticism of the manuscript and to Mr. J. M. Airth for the Statistical Analyses of the data.

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Paper Chromatographic Identification of Thiourea in Extracts of Oranges

By W. P. McKINLEY and ROSE YASIN (Food and Drug Directorate, Department of Health and Welfare, Ottawa, Ontario, Canada)

This paper outlines a procedure to identify thiourea in extracts of oranges in the presence of pesticides and other interferences.

METHOD

Apparatus

(a) *Chromatographic jars, spray bottles, and accessories.*—See McKinley and Mahon (1).

Reagents

(a) *Mobile phase.*—*n*-Butanol saturated with water. Prepare by adding equal volumes of water and *n*-butanol to a separatory funnel. Shake vigorously, allow to separate overnight, and discard aqueous phase.

(b) *Thiourea standard solution.*—B.D.H. reagent grade; 0.04*M* in acetone.

(c) *Thiran.*—Pure (E. I. duPont de Nemours and Co., Inc.). 0.02*M* solution in acetone.

(d) *Sodium azide.*—Practical (Eastman Organic Chemicals). 3% w/v aqueous solution.

(e) *Iodine.*—Crystalline; 25 g stored in large desiccator.

(f) *Starch.*—Dissolve 3 g soluble starch (B.D.H., Analar) in 200 ml hot water (80°C) and add 100 ml saturated aqueous solution of sodium chloride to give a 1% (w/v) solution.

(g) *Grote's reagent.*—See Allport and Keyser (2).

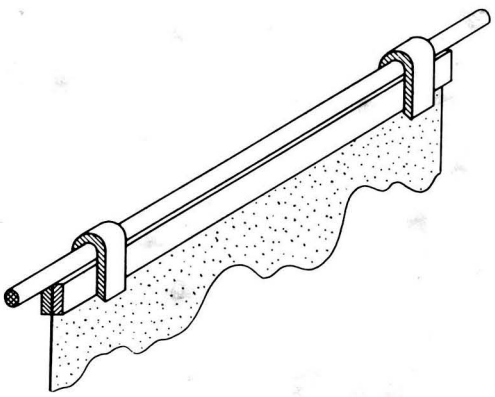

Fig. 1—Paper holder consisting of 2 pieces of Perspex 3 mm × 15 mm and 21 cm long.
The two clips holding the circular glass rod to the two strips of Perspex were heated in an oil bath and bent to the desired shape.

Extraction of Residue and Removal of Solvent

Extract a 900–1100 g sample of whole oranges with 500 ml distilled water by the roller stripping procedure (1). Filter extract through a medium porosity filter and place in a 1000 ml round-bottom flask fitted to a condenser. Remove water under reduced pressure at 40–50°C with a water aspirator until the volume is approximately 30 ml. Transfer remaining solution to a 100 ml round-bottom flask, rinse the 1000 ml flask with three 5 ml portions of acetone, and reduce volume to near dryness. Dissolve the soluble portion of the residue in 10 ml acetone and rinse flask twice with 3 ml portions of acetone. Filter solution through a medium porosity sintered glass filter and remove solvent under nitrogen at 40–50°C.

Paper Chromatographic Analyses

Redissolve residue in 5 ml acetone and apply 5 microliters of the solution to an unwashed sheet of 8½″ × 8½″ Whatman No. 1 filter paper. Apply a five microliter aliquot of the standard thiourea and the same quantity of thiram standard to the paper sheet. Prepare a second paper in the same manner. Place each paper sheet in a clamp as shown in Fig. 1 and develop in the *n*-butanol system. Spray one paper (Fig. 2) with the sodium azide reagent, subject to iodine vapors, and spray with the starch solution as described for the metal-containing dithiocarbamate fungicides (3). Spray the other paper with Grote's reagent (4) as a confirmatory test.

In the test for dithiocarbamates fiber glass paper was used, and the paper was impregnated with formamide to retain the iodine. This was not necessary in the present work because Whatman No. 1 paper was employed.

Results and Discussion

Chromatographic results are best explained by referring to the chromatogram in Fig. 2. The spot in the upper portion at A is the thiram standard and the spot about one-third of the way up the paper at A is the

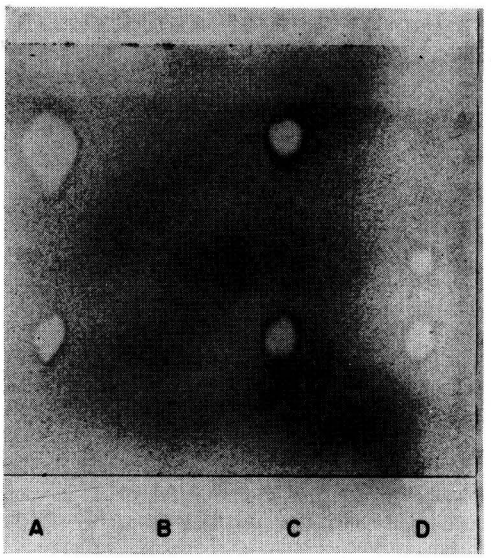

Fig. 2—Chromatogram developed with sodium azide, iodine, and starch reagents.
A: thiram standard (upper spot), thiourea standard (lower spot); B: crop blank; C: extract of organges which contained thiram (upper spot), thiourea (lower spot), biphenyl, o-phenyl phenol, glyodin, and captan (not showing); D: extract of oranges containing nabam (upper spot), thiourea (lower spot), thiram, nabam, ferbam, maneb, ziram, and zineb (not showing).

thiourea standard. B is a 5 microliter aliquot from the extract of 1000 g of oranges which were free of pesticides, and is referred to as the crop blank. There was no interference from extraneous material in these extracts. C in Fig. 2 represents an extract of 1000 g of oranges which were rotated with 500 ml of water in a bottle containing 10 mg of each of the following: thiourea, thiram, biphenyl, *o*-phenyl phenol, glyodin, and captan. Thiram and thiourea were the only two compounds extracted with water as the solvent.

The portion of the chromatogram labeled D in Fig. 2 is an aliquot of another extract of 1000 g of oranges which were extracted in a jar containing 10 mg of each of the following: thiourea, thiram, ferbam, nabam, maneb, ziram, and zineb. The spot one-third of the distance up the paper is thiourea, and the spot half the distance up the paper is nabam. An interesting observation is that thiram was not extracted when added along with the metal-containing dithiocarbamates. It would appear that the thiram may have complexed with one of these compounds in such a manner as to be rendered water-insoluble. These observations were confirmed with other extracts, indicating that thiram and nabam are the only two of the compounds tested which might extract and give the same reaction as thiourea. These three materials are resolved readily by the chromatographic system.

The presence of thiourea can be confirmed by spraying a second chromatogram fairly heavily with Grote's reagent, which produces a blue color for both nabam and thiourea but not for thiram. Kjaer (3) detected a number of N-monosubstituted thioureas with Grote's reagent after heating the paper at 100°C, but the color faded in a few days. Kjaer states that the color may be eluted and measured spectrophotometrically with an accuracy of ± 5%. Although the test may be less sensitive, the papers were not heated in the present study, and the color was stable for several weeks.

The present paper deals only with extracts of whole oranges. Winkler (5) has used Grote's reagent for the quantitative determination of thiourea in orange juice and frozen peaches and has investigated extraction and clean-up procedures as well as the reaction with the reagent.

Acknowledgments

Thanks are extended to Mr. M. Osadchuk of this Department for his suggestion of the paper clamp used in Fig. 1, and to Dr. D. G. Chapman and Constance Webster for constructive criticism of this report.

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AN EXPERIMENT IN THE SAMPLING AND ANALYSIS OF BAGGED FERTILIZER

Sponsored by The National Plant Food Institute (1700 K Street, N.W., Washington 6, D.C.)
Under the direction of Vincent Sauchelli, *Chairman,* National Plant Food Institute Task Force.
Report prepared by Acheson J. Duncan, The Johns Hopkins University.

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INTRODUCTION AND ACKNOWLEDGMENTS

Aware of the difficulties and problems of sampling which confront the fertilizer industry, the National Plant Food Institute (NPFI) decided to act upon a recommendation made in January, 1956 by the Soils, Water, and Fertilizer Advisory Committee to the U.S. Department of Agriculture to the effect that consideration be given to improving methods for fertilizer quality control. Accordingly, the NPFI appointed a Task Force representing the Association of Official Agricultural Chemists (AOAC), the Association of American Fertilizer Control Officials (AAFCO) and the industry. This group met in Washington on March 23, 1956 to discuss and design a comprehensive research project aimed at improving procedures of sampling and sampling instruments. The NPFI Task Force approved this project in the fall of 1956.

The following report on the NPFI project is a contribution in the general field of sampling as applied to bagged fertilizers. The application of modern statistical principles to the design of the experiment and the analysis of the experimental data is the significant feature of this work.

The Task Force was made up as follows:

National Plant Food Institute
Task Force on Chemical Control
Research Project

Vincent Sauchelli, *Chairman,* National Plant Food Institute.

J. R. Archer, Chief Chemist, Plant Food Division, International Minerals and Chemical Corporation.

E. D. Crittenden, Consultant, Nitrogen Division, Allied Chemical Corporation.

W. L. Hill, Agricultural Research Service, U. S. Department of Agriculture.

Stacy B. Randle, State Chemist, New Jersey Agricultural Experiment Station.

Maurice B. Rowe, Superintendent of Motor Fuels and Fertilizers, Division of Chemistry and Foods, Virginia Department of Agriculture.

M. D. Sanders, Director of Research and Development, Agricultural Chemicals Division, Swift and Company.

R. P. Thornton, Thornton Laboratories, Inc.

Edwin C. Kapusta, U. S. Potash Company Division.

C. H. Perrin, Research Chemist, Canada Packers, Limited.

Bruce Poundstone, Head, Department of Feed and Fertilizer Control, University of Kentucky.

LIST OF TABLES

F. W. Quackenbush, Head, Department of Biochemistry, Purdue University.

H. J. Webb, Head, Agricultural Chemistry Research, Clemson Agricultural College.

Special Counsellors to Task Force:

Dr. W. Edwards Deming, New York University.

Dr. A. J. Duncan, The Johns Hopkins University.

The experiment was under the general direction of Vincent Sauchelli who makes the following acknowledgments:

"We are grateful to Messrs. W. L. Hill, Milton Norland, and Carl Eriksson, U.S. Department of Agriculture, ARS, Fertilizer and Lime Research Division, for much help in editing and checking the mass of data. Dr. A. J. Duncan of The Johns Hopkins University devoted considerable time and effort in assembling, analyzing the tabulated chemical and physical data, and in writing this report. Mr. Albert Spillman, Manager, Fertilizer Manufacturing Cooperative, gave unstinted cooperation during the fertilizer-sampling phase. Messrs. Stacy B. Randle, Maurice Rowe, and Hugh Webb, State Chemists at New Jersey, Virginia, and South Carolina, respectively, provided generously of their time and laboratory facilities in the analysis of the large number of samples involved in the experiment.

"To all those on the Task Force who helped with their counsel in organizing the work and interpreting the results of the experiment go our grateful thanks."

SUMMARY OF IMPORTANT CONCLUSIONS

(*1*) The experiment offers very little evidence of the existence of systematic variations in the use of the three sampling instruments under study. Out of 15 comparisons between the three instruments only one yielded a statistically significant difference; this was in the per cent nitrogen obtained in samples from the granulated 8-16-16 fertilizer. That this is evidence of a real difference, however, is questionable. Since all statistical tests were carried out at the 0.05 level, one significant difference in 15 is about what would have been expected on a chance basis if no real differences at all existed.

(*2*) There is some evidence of systematic variations attributable to the men taking the samples. The results suggest that a man may introduce a small systematic effect in the results obtained from sampling, but there is no evidence that these systematic effects vary with the instrument used.

(*3*) There is definite evidence that samples taken by sampling tubes yield mean results that are different from those obtained by riffling. The systematic variations are especially prominent with respect to the per cent potash obtained, the riffles in some fertilizers yielding a lower per cent of this chemical element.

(*4*) Although sampling by tubes and sampling by riffling appear in certain cases to yield different mean results, the reproducibility (i.e., the precision) of results obtained by the two methods appears to be equally good. Although the relative reproducibility varies strikingly from one set of conditions to another, there is no consistent pattern that might warrant any conclusions as to the greater precision of one of the two methods of sampling.

(*5*) The three laboratories cooperating in the experiment showed definite systematic variations that are sizeable in amount. Although the mean differences between laboratories may have been partly due to atmospheric and other differences that also cause variations from day to day in the same laboratory, the size of the interlaboratory differences is so much greater than the day-to-day variation within the same laboratory, revealed in another part of the experiment, that it can hardly be questioned that mean differences between laboratories exist.

(*6*) The experiment yields evidence of day-to-day differences within the same laboratory that are over and above differences that can be explained by errors in laboratory tests made the same day. The net day-to-day variations appear in general to be of the same order of magnitude as the within-day variations.

(*7*) There is evidence that if segregation in the bin can be eliminated and its results avoided, bag-to-bag variation can be markedly reduced. Variations attributable to the bagging operation itself show little evidence of systematic influences.

(*8*) Net components of variance for sampling and testing are derived from the results of the experiment and are put together to indicate the order of magnitude of tolerances for tests of the various chemical elements. These tolerances are much larger than those offered by Miles and Quackenbush [*This Journal,* **38,** 108 (1955)] primarily because of inclusion of data on interlaboratory variation which were not available in their studies. In view of this evidence that substantial interlaboratory variation exists, experiments should be performed with a larger number of laboratories in order to obtain a reliable measurement of its magnitude.

CHAPTER I

THE PLANNING AND CONDUCT OF THE NPFI EXPERIMENT

1. Purpose and Scope of the Experiment

The committee that met in 1956 set forth as a principal purpose of the experiment the determination of the existence and amount of systematic variation in different methods of sampling bags of fertilizer. It singled out for study the comparison of three specific sampling instruments as well as the comparison of sampling by instruments with sampling by riffling. In designing the experiment to accomplish these comparisons, it turned out to be desirable to have information on whether taking a series of samples by the same instrument from the same bag in the same position produced a systematic trend or order effect. The determination of order effects thus became a secondary objective of this part of the experiment. Another supplementary objective was to discover whether the men taking the samples showed systematic effects in their use of the sampling instruments.

A second fundamental purpose of the experiment was to determine the existence and amount of systematic laboratory errors. The principal objective was to make interlaboratory comparisons but, as conducted, the experiment also yielded information on day-to-day variations within the same laboratory. Although the experiment was not initially designed to measure variations between chemical determinations run by the same laboratory on the same day, a supplementary experiment was eventually conducted to yield this information.

A final purpose of the experiment was to measure variation from bag to bag and within bag. As conducted, the bags were taken from the bagging operations at random so that the experiment yielded information on variations from the beginning to the end of the bagging of the manufacturer's lot, as well as information on variations between bags within a given shipment.

The experiment was designed so that in the case of some fertilizers it was possible in a single comprehensive analysis to isolate the various types of variation and ultimately to compute "error tolerances." These may be useful to manufacturers and state regulatory agencies.

The instruments selected for study were a standard single tube, a double tube, and a special tube designed by J. R. Archer.[1]

[1] Chief Chemist, International Minerals & Chemical Corp. Plant Food Division.

These tubes are designated *x*, *y*, and *z*. They had the following characteristics:

Instrument	*Width of Slot*	*Diameter of Core*
Single (*x*)	½″	9/16″
Double (*y*)	½″	¾″
Archer (*z*)	⅞″	1″

The Archer tube is shown in Figs. I-1 and I-2.

Four fertilizers, designated as A, B, C, and D, were studied, and are described in Table I-1.

Table I-1. Chemical contents of fertilizers used (per cent)

Fertilizer	Nitrogen	Phosphorus (P_2O_5)	Potash (K_2O)	Type
A	10	10	10	Granulated
B	0	20	20	Powdered
C	5	10	10	Powdered
D	8	16	16	Granulated

All fertilizers were made by the Fertilizer Manufacturing Cooperative, Inc., Baltimore. As noted above the bags used in the experiment were selected as the fertilizer was being bagged. The bags were stored at the plant and the instrument samples and riffles were taken there in a single day. All samples were transported in plastic bags to the various laboratories.

Three of the men who took the samples were state inspectors experienced in sampling fertilizer in bags. The fourth was J. R. Archer, the designer of instrument *z*. The three state inspectors cooperated in that part of the experiment designed to test for the existence of order effects. Mr. Archer, an "extra" man, took additional samples from each bag at random. (*See* "Comparison of Instruments and Men" under Section 2, immediately following.)

The three state inspectors, numbered 1, 2, and 3, are referred to by these numbers. Mr. Archer is referred to as man No. 4.

The state laboratories of Virginia, New Jersey, and South Carolina analyzed the various samples taken as part of the experiment. These are designated as Laboratory 1, Laboratory 2, and Laboratory 3, respectively.

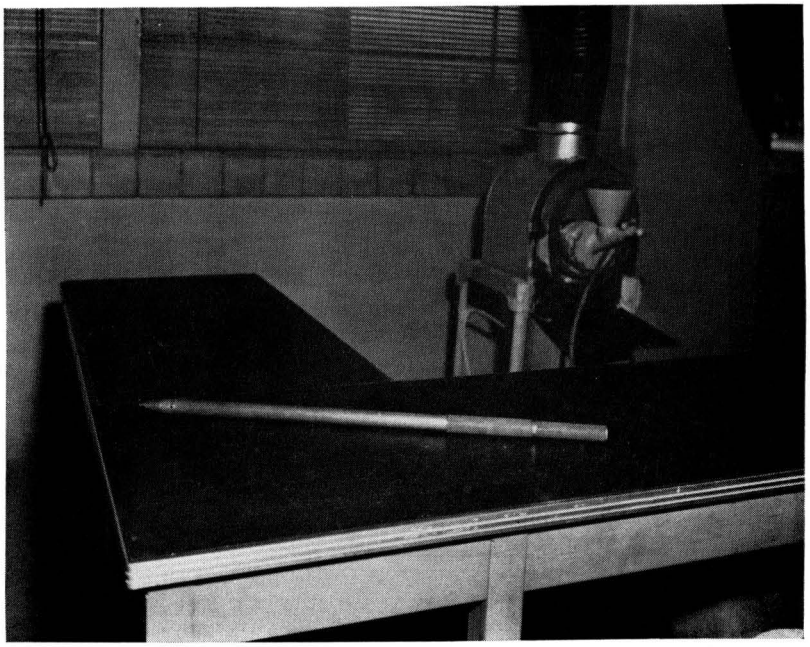


Fig. I-1—Archer sampling tube.

Fig. 1-2—Archer sampling tube.

2. Design of the Experiment

Comparison of Instruments and Men

Since it was believed that any existent systematic effects of instruments or men would be relatively small, it was necessary that the error variance for testing for these systematic effects be as small as possible. Therefore samples for comparing either instruments or men were taken from the same bag, since it was believed that bag-to-bag variation would be much greater than within-bag variation. Also the samples were taken as much as possible from the same part of the bag. It was feared, however, that taking repeated samples from the same bag and same position might introduce order effects that would become confounded with instrument-effects and man-effects. Some confounding of order-effects with man-effects might be allowed since this was not a primary objective of the experiment, but no confounding of order-effects with instrument-effects could be tolerated.

The Latin square was a natural design for the study and elimination of order effects. A unit experiment was thus planned so that in sampling a single bag three men

would use three different instruments in three different orders in such a way that each instrument would be used once by each man and once in each order. This Latin square design was replicated for three separate bags although the exact design was not the same for all bags. Examples of Latin squares used in the sampling are in the right-hand column. A certain amount of confounding was allowed in the design in that after six samples had been taken diagonally from one corner of a bag, the next three were taken diagonally from the opposite corner. Since each man took his three samples, one with each instrument, before the next one took his turn, in the case of a single bag the variation between men was confounded with any existent effect attributable to position. There was no confounding, however, of position effect with instrument effects. To allow ultimately for a more extended analysis if the order effects should prove to be non-significant, a fourth man (who was always the same man) was allowed to take his three samples from each bag in an order assigned at random with respect to the three other men.[2]

As noted, this part of the experiment yielded samples from three different bags of fertilizer of the same grade. Those from the first bag were sent to one of the three different state laboratories, those from the second bag to another of the three laboratories, and those from the third bag to the last laboratory. The bag-laboratory combinations were thus "blocks" for replication of the unit experiment. All samples were subjected to chemical analysis and those of the granulated fertilizers to sieve analysis.

Instruments: x, y, z

Block 1

Order

Men	1	2	3
1	*z*	*y*	*x*
2	*x*	*z*	*y*
3	*y*	*x*	*z*

Block 2

Order

Men	1	2	3
1	*x*	*z*	*y*
2	*y*	*x*	*z*
3	*z*	*y*	*x*

Block 3

Order

Men	1	2	3
1	*x*	*y*	*z*
2	*y*	*z*	*x*
3	*z*	*x*	*y*

Comparison of Methods of Sampling

A second part of the experiment was designed to show how samples obtained from a bag of fertilizer by instruments compared on the average with samples obtained by "riffling." This part of the experiment was again divided into three blocks; a block consisted of a set of 7 bags all samples of which were analyzed by a single laboratory.

A man and sampling instrument were selected at random from the four men and three instruments used in the first part of the experiment. This man with the selected sampling instrument took two samples from each of the seven bags, one diagonally from the right-hand corner of the bag and one diagonally from the left-hand corner. The

[2] *See* Note 2 of the Appendix for full details of how the various instruments, men, and laboratories were assigned tasks at random.

remainder of the 80 pound bag was in each case run through a large Tyler riffle which split the contents in a 15:1 ratio (Fig. I-3). The 15/16 portion was riffled again to yield two approximately 1/16th riffles per bag. All fourteen samples of the given block were tested by the associated laboratory. The procedure was repeated for the other two blocks.

Interlaboratory Comparisons

The three state laboratories that carried out the chemical analyses for the first two parts of the experiment also participated in interlaboratory comparisons. The design for this was as follows: Seventy-five grams from each of the twelve instrument samples that formed a single "block" in the first part of the experiment were pooled and ground by the laboratory concerned to pass 35 mesh. This ground fertilizer was then divided by a laboratory sample riffle into eight parts. Two of these eight parts were selected at random for subsequent chemical analysis by the preparing laboratory. Two others were selected at random and sent to a second laboratory, and a third set of two was selected at random and sent to the third laboratory. This was done by all three laboratories for the bags they analyzed, so that

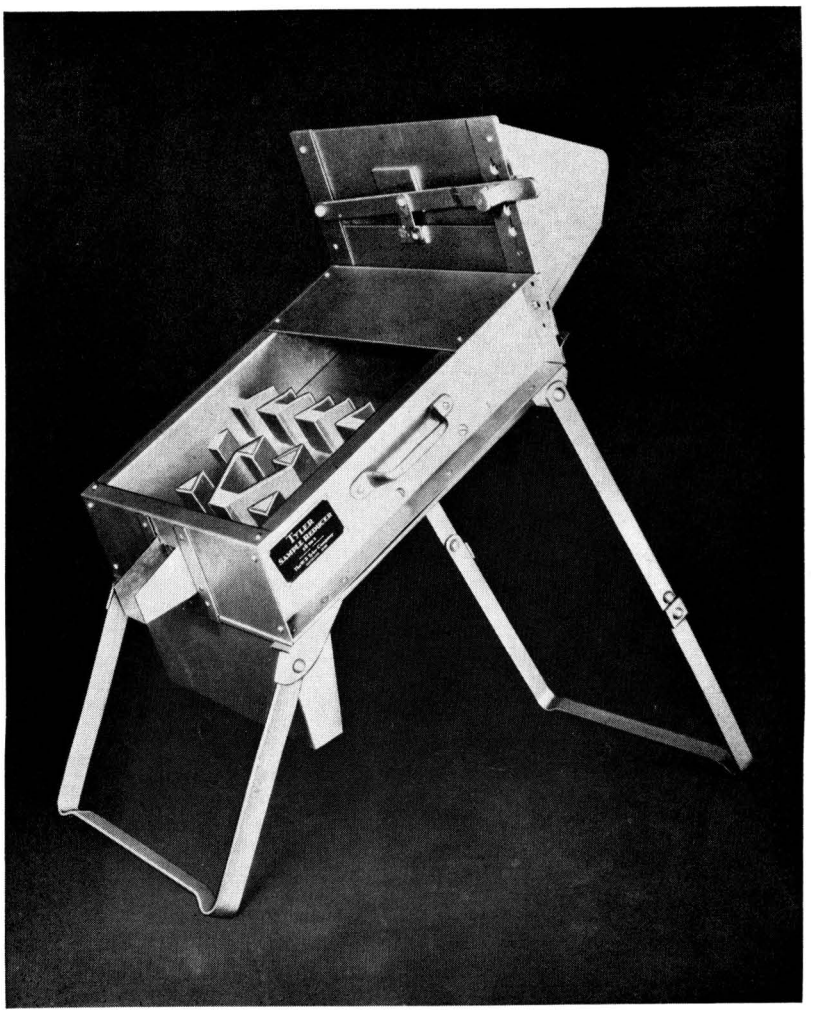


Fig. I-3—Tyler riffle or sample reducer used in the experiment.

ultimately each laboratory tested two samples from a given bag. All six samples were made "homogeneous" by grinding to 35 mesh and the whole was repeated for three bags.

Intra-Laboratory Variations

A fourth part of the experiment was designed to measure what might be called within-laboratory test error. Previous studies had reported on variation between "duplicates," and it was believed that it might be interesting for the National Plant Food Institute experiment to study variation between tests run by the same laboratory on the same sample a week apart. Therefore, on a certain number of samples that entered into other parts of the experiment, the laboratories were directed to run a second test one week following the first test.

This feature of the experiment was added without much discussion by the planning committee and it was not until after the preliminary results were analyzed and presented for review that the statistician in charge of the analysis realized that his "model" of laboratory variations was incorrect. The "duplicates" run in earlier experiments were tests on the same sample, but were run on different days. It also turned out that the blocks of 14 chemical determinations run in this part of the experiment were generally run by the laboratories in a single day. The mean difference between tests now and tests a week later thus yielded only a single measure of the day-to-day effect[3] for each laboratory, and this was probably a measure of the same variation reported in earlier experiments as variation between duplicates. Fortunately one of the laboratories had samples of the original fertilizer on hand and a supplementary experiment was designed to measure both day-to-day variation and variation between duplicates within the same day. This consisted in having each of the three laboratories run two tests a day on the same sample for each of seven days.

The overall results thus provided two independent measures of day-to-day variation within the same laboratory and two independent measures of within-day variation. One of the latter was a direct measure of this variation. The other was a measure based on the residual variation in the block of 28 tests called for in the original experiment after the bag-to-bag and day-to-day variation had been eliminated.

Bag-to-Bag Variations

As noted above, the bags selected for study in the experiment were selected at random from the manufacturer's bagging operations. The amount of fertilizer in the bin awaiting to be bagged on order was given to the statisticians considerably in advance of the time planned for taking the samples. The number of 80 pound bags that would result from bagging this fertilizer was estimated. A random sample of potential bags was selected by assigning numbers to the bags in order of their production and the selection of a sample by use of a table of random numbers.[4] The bags were actually selected by reference to these numbers under the supervision of an operator trained for the job and working in close cooperation with the manufacturer. Not only was a random sample from the bagging operations thus assured but the particular place of a bag in the order of production was known so that manufacturing variation over time could be studied. Since the manufacturer bagged and shipped only on order, it was also possible to tell from his records which bags in the sample came from which shipment.

The Over-all Adequacy of the Experiment

By generally running the experiment in triplicate (e.g., one bag or a separate set of bags for each laboratory) it was judged by the statisticians that the experiment would have sufficient precision to discover differences of importance if they existed.

[3] This was still probably a "day-to-day" effect even though the tests were run a week apart. It will be designated as such in the remainder of the report.

[4] Actually the sampling was somewhat more complicated than this since the bags were loaded on six-bag skids immediately following bagging and the sampling was related to the skids. For a full description of the method of sampling, *See* Appendix, Note 1.

Estimates of the actual adequacy of the experiment are given in the accompanying tables.[5]

In comparing the instruments the experiment was judged to have had a probability of roughly 0.90 of discovering differences of the magnitude shown in Table I-2.

In comparing the laboratories the experiment was judged to have had a probability of roughly 0.90 of discovering differences of the magnitude shown in Table I-3.

Table I-2. Standard deviation of differences between instruments having a high probability of detection by the experiment

Fertilizer and Chemical Element		Standard Deviation (%)
A.	10–10–10, granulated	
	N	0.07
	P	0.05
	K	0.11
B.	0–20–20, powdered	
	P	0.22
	K	0.11
C.	5–10–10, powdered	
	N	0.04
	P	0.07
	K	0.12
D.	8–16–16, granulated	
	N	0.15
	P	0.13
	K	0.20

[5] *See* A. J. Duncan, Quality Control and Industrial Statistics, 2nd Ed., pp. 525. The standard deviations of the random term were estimated from the experimental results.

Table I-3. Standard deviation of differences between laboratories having a high probability of detection by the experiment

Fertilizer and Chemical Element		Standard Deviation (%)
A.	10–10–10, granulated	
	N	0.19
	P	0.11
	K	0.07
B.	0–20–20, powdered	
	P	0.17
	K	0.23
C.	5–10–10, powdered	
	N	0.05
	P	0.11
	K	0.14
D.	8–16–16, granulated	
	N	0.09
	P	0.11
	K	0.11

The standard deviation is taken here to be the root mean square of the deviations of the instruments from a common mean. For example, suppose instrument x has a systematic variation of +0.02, instrument y a systematic variation of +0.09, and instrument z a systematic variation of −0.11. (The systematic variations must necessarily sum to zero, since they are all viewed as relative.) Then, the root mean square or standard deviation will be:

$$\sigma = \sqrt{\frac{0.02^2 + 0.09^2 + 0.11^2}{3}} = 0.08$$

CHAPTER II

COMMENTS ON THE STATISTICAL RISKS OF THE EXPERIMENT

In this report results are presented usually in the form of pertinent means, differences between individual results and means of these differences, ranges of means, and ranges of differences. Both tabular and graphic methods of presentation are employed. In most cases the results will be readily understood by the general reader.

In the Appendix the results are subjected to more sophisticated statistical analysis. This will be of interest to those trained in statistical procedures. Whereas the technical analyses are not presented in the body of the report, the general conclusions are. This generally takes the form of reporting on the "statistical significance" of the results.

In technical statistical language a result is said to be "statistically significant" if it cannot readily be explained by chance. We usually start with the hypothesis that things are *not* different, the "null hypothesis" as it is called. If the results obtained in an experiment are in sufficiently close accord with those expected on the basis of the null hypotheses, we continue to accept this hypothesis, i.e., we say the results are not "statistically significant." If however the results differ from those expected to the extent that they cannot reasonably be considered chance deviations, then the null hypothesis is rejected; we say the results are statistically significant and conclude that a real difference exists.

In carrying out this statistical analysis it is necessary to decide how large a deviation from the expected will be considered as probably not due to chance. Generally, if a deviation is so large that the probability of as large or a larger value occurring by chance is less than 0.05, this deviation is said to be statistically significant at the 0.05 level. In this report, tests are usually conducted at an 0.05 level, and unless it is otherwise stated, if a result is said to be "statistically significant," it means that the probability of as large or larger deviation occurring by chance is less than 0.05. In some cases it is pointed out that results are significant at the 0.01 level and in a few cases at the 0.001 level.[6]

For the most part the statistical analyses of the Appendix take the form of what is called an "analysis of variance." In simple terms this consists in comparing the variation in a set of means with some measure of chance variation that is independent of the variation in the means themselves. When the variation in a set of means is reported in the body of the text as being "statistically significant," it is to be inferred that the variation in the set of means is such that in the light of the measure of chance variation available, the probability of the variation in the means occurring as a mere result of chance and not a real difference is less than 0.05.

In designing the experiment the statisticians sought to get measures of chance variation that were sufficiently small to show up real differences of importance. If enough tests can be run, a well-designed experiment will be able to discover differences of minute size. To detect such differences, however, the cost would be very high and the differences found might not be worth the expense of their discovery. In this experiment, it was decided that sufficient time and money were available to detect differences between instruments, laboratories, etc., that were equal approximately to the standard deviation of samples taken from the same bag or some composite mix. The experiment was so designed that differences between instruments, laboratories, etc. of this order of magnitude would have roughly a 0.90 chance of being detected if they existed. Estimates of the actual adequacy of the experiment were given above in Chapter I, "The Overall Adequacy of the Experiment" under Section 2.

Part of the report presents estimates of various components of variation, e.g., variation due to sampling, to laboratory test error, etc. In general the presentation of

[6] In the tables in the Appendix, results significant at the 0.05 level are marked with a single asterisk; those significant at the 0.01 level or lower are marked with two asterisks.

these estimates is accompanied by a statement of the 0.95 confidence limits for the true value. The purpose in giving confidence limits is to give the reader some idea as to the reliability of the estimates presented. If it is said, for example, that a certain component of variation is estimated at 0.06 and that 0.95 confidence limits are 0.05-0.08, this means that the best estimate of the component obtained from the experiment is 0.06. The true value, however, may well deviate from this and to indicate the likely range of this deviation we say that the range 0.05 to 0.08 will have a 0.95 chance of containing the true value. In other words, the statement that the true value lies in the range 0.05 to 0.08 has a 0.95 chance of being correct. In some cases the amount of data in the experiment on which the confidence limits are based is very small, with the consequence that the limits are very wide. It will be generally noted that when the data are meager, confidence limits for components of variance are highly unsymmetrical about the estimated value, the upper limit being much further above the estimated value than the lower limit is below it. It will also be noted that except for residual components the confidence limits are based upon estimates of other components that are themselves in error, so that for higher order components the confidence limits are very rough. (*See* Appendix, Note 17). It is believed, however, that even rough estimates of reliability are better than none at all.

CHAPTER III

SYSTEMATIC EFFECTS AND RELATIVE PRECISIONS OF METHODS OF SAMPLING

This chapter presents the results of the experiment pertaining to the systematic effects and relative precisions of the various methods of sampling fertilizer in bags. Chapter IV discusses systematic effects and chance variations due to sampling the fertilizer in the laboratory after it has been ground to pass a 35-mesh for chemical analysis.

1. Effects of Order of Sampling Bags

The experiment was so designed that it permitted a test of order effects prior to a general analysis of instrumental effects. Tables III-1 and III-2 present the results pertaining to order effects. It will be recalled that the experiment called for three samples to be taken in succession from a bag from the same general position (e.g., left diagonal or right diagonal). The means shown in Tables III-1 and III-2 are means over several samples of the three orders of sampling from the same position in the same bag. Table III-1 shows sieve weights and Table III-2 chemical analyses.

It will be noted that for both sieve weights and chemical analyses the variation in mean results by order of sampling is generally quite small. Furthermore the Appendix shows that none of the variations presented in Tables III-1 and III-2 are statistically significant. (*See* Note 7 of the Appendix). The experiment thus gave no evidence of any order effects.

2. Systematic Variations in the Sampling Instruments

The experiment called for each of four men to take samples from the same bag of

Table III-1. Effects of the order of sampling the bags: Sieve analysis

(Means of nine samples in each order, in per cent of total weight retained on each sieve)

Fertilizer and Mesh[a]	Order 1	Order 2	Order 3	Range or Maximum Difference	Statistically Significant?
A. 10–10–10, granulated					
Mesh 6	8.7	9.1	8.6	0.5	No
Mesh 14	69.5	67.5	70.6	3.1	No
Mesh 24	17.9	18.4	17.7	0.7	No
D. 8–16–16, granulated					
Mesh 6	8.3	7.8	8.7	0.9	No
Mesh 14	58.6	57.2	59.4	2.2	No
Mesh 24	17.0	18.7	17.5	1.7	No

[a] For standard Tyler sieves.

Table III-2. Effects of the order of sampling the bags: Chemical analysis

(Means of nine samples in each order, in per cent)

Fertilizer and Element	Order 1	Order 2	Order 3	Range or Maximum Difference	Statistically Significant?
A. 10–10–10, granulated					
Nitrogen	9.91	9.89	9.93	0.04	No
Total phosphorus	11.16	11.22	11.17	0.06	No
Available phosphorus	11.00	11.06	11.02	0.06	No
Potash	10.86	10.68	10.88	0.20	No
B. 0–20–20, powdered					
Total phosphorus	21.23	21.21	21.43	0.22	No
Available phosphorus	20.59	20.57	20.79	0.22	No
Potash	20.11	20.20	20.11	0.09	No
C. 5–10–10, powdered[a]					
Nitrogen	5.93	5.88	5.85	0.08	No
Total phosphorus	11.17	11.13	11.13	0.04	No
Available phosphorus	10.48	10.43	10.45	0.05	No
Potash	11.30	11.30	11.32	0.02	No
D. 8–16–16, granulated					
Nitrogen	8.28	8.20	8.38	0.18	No
Total phosphorus	17.70	17.70	17.76	0.04	No
Available phosphorus	17.13	17.13	17.21	0.08	No
Potash	16.73	16.61	16.52	0.21	No

[a] Based on 6 samples instead of 9.

fertilizer with each of the three instruments. This was replicated three times using different bags of fertilizer of the same grade. The samples from the first replicate were sent to Laboratory 1 for test, those from the second replicate to Laboratory 2, and those from the third to Laboratory 3. This experiment was repeated for each of the four grades of fertilizer. All samples were subjected to chemical analysis and the two granulated fertilizers were subjected to sieve analysis.

Sieve Analysis

Table III-3 summarizes the results of the

sieve analysis for the two granulated fertilizers. It will be noted that in both Fertilizers A and D instrument *z* shows a tendency to take in a higher percentage of larger particles than the other instruments and a lower percentage of smaller particles. The analysis of variance presented in Table A 3 of the Appendix shows that these differences between instruments are statistically significant. A significant difference is thus demonstrated between the instruments with respect to selection of particle size.

As noted above no sieve analysis was made of Fertilizers B and C.

Chemical Analysis

Table III-4 summarizes the results of the

Table III-3. Effects of sieve analysis averaged for instruments

(Means of 12 samples, in per cent of total weight retained on each sieve)

Fertilizer and Mesh[a]	Instruments			Range or Maximum Difference	Statistically Significant?
	x	*y*	*z*		
A. 10–10–10, granulated					
Mesh 6	8.7	7.5	10.2	2.7	At 0.001 level
Mesh 14	68.2	68.0	68.9	0.9	No
Mesh 24	19.1	19.4	16.9	2.5	At 0.05 level
D. 8–16–16, granulated					
Mesh 6	8.3	7.2	9.5	2.3	At 0.05 level
Mesh 14	58.1	57.8	59.2	1.4	No
Mesh 24	18.0	18.5	17.3	1.2	No

[a] For standard Tyler sieves.

Table III-4. Results of chemical analysis of various instrument samples

(Means of twelve samples, with each instrument, in per cent)

Fertilizer and Element	Instruments			Range or Maximum Difference	Statistically Significant?
	x	*y*	*z*		
A. 10–10–10, granulated					
Nitrogen	9.88	9.92	9.89	0.04	No
Total phosphorus	11.13	11.17	11.16	0.03	No
Available phosphorus	10.99	11.03	11.00	0.04	No
Potash	10.83	10.81	10.80	0.03	No
B. 0–20–20, powdered					
Total phosphorus	21.23	21.43	21.39	0.20	No
Available phosphorus	20.58	20.79	20.73	0.21	No
Potash	20.16	20.18	20.03	0.15	No
C. 5–10–10, powdered					
Nitrogen	5.55	5.53	5.58	0.05	No
Total phosphorus	11.30	11.25	11.30	0.05	No
Available phosphorus	10.57	10.53	10.60	0.07	No
Potash	11.40	11.50	11.33	0.17	No
D. 8–16–16, granulated					
Nitrogen	8.30	8.16	8.44	0.28	At 0.05 level
Total phosphorus	17.65	17.63	17.79	0.16	No
Available phosphorus	17.09	17.07	17.22	0.15	No
Potash	17.70	17.74	17.41	0.33	No

chemical analysis for all four fertilizers. In general it shows very little average difference between the three instruments; the highest of the maximum mean differences is 0.33% and all but three are equal to or less than 0.20%. In Fertilizers B, C, and D there is a tendency for instrument *z* to yield a slightly lower per cent potash than the other instruments; this is not true, however, of Fertilizer A. This result is in agreement with the results of the sieve analysis which suggested that instrument *z* picks up a smaller percentage of the smaller particles than instruments *x* and *y*.

The sample mean differences between the instruments are small, however, compared to the sampling and test errors, and consequently only one set of differences is statistically significant. These are the differences between the nitrogen means of Fertilizer D. (*See* Note 8 of the Appendix.) The mean nitrogen content of samples drawn with instrument *x* is midway between the means for *y* and *z*. Actually, the difference between the nitrogen means of Fertilizer D is no greater than the difference between the potash means of Fertilizer D and little greater than the difference between the phosphorus means of Fertilizer B. The sampling and test variance, however, are so much greater for potash of Fertilizer D and phosphorus of Fertilizer B that the differences are not statistically significant.

Altogether, a total of 15 comparisons is made of the three instruments and the only significant difference is that between the nitrogen means of Fertilizer D. Since the statistical tests are all run at the 0.05 level, i.e., the false acceptance of the existence of a real difference may occur in 1 out of 20 comparisons, the occurrence of one significant result in 15 analyses is only a little more than what might be expected if no real differences existed at all. It is to be concluded therefore that the over-all experiment offers little evidence of any real differences among the three instruments with respect to chemical content.

3. Systematic Variations of the Men Taking the Samples

The mean sieve results and the mean chemical determinations for the four men who did the sampling for the comparison of instruments are shown in Tables III-5 and III-6. Two sets of differences between sieve means are reported to be statistically significant at the 0.05 level and one set of differences between means of chemical determinations are reported statistically significant at the 0.01 level and another at the 0.001 level.

Table A 4 of the Appendix also shows that in four cases there are statistically significant "interactions" between the men and the "blocks" of samples sent to the various laboratories. This means that in one block the differences between the mean results for the four men were significantly different from the differences between the mean results for

Table III-5. Results of the sieve analysis classified by men taking the samples

(Means of 12 samples in per cent retained on each sieve)

Fertilizer and Mesh[a]	Men 1	2	3	4	Range or Maximum Difference	Statistically Significant?
A. 10–10–10, granulated						
Mesh 6	6.1	6.9	6.9	6.5	0.8	No
Mesh 14	52.8	52.4	50.4	49.4	3.4	At 0.05 level
Mesh 24	13.2	12.7	14.5	14.8	2.1	At 0.05 level
D. 8–16–16, granulated						
Mesh 6	6.4	6.2	6.0	6.3	0.4	No
Mesh 14	45.0	42.7	43.8	43.6	2.3	No
Mesh 24	13.0	13.6	13.3	13.8	0.8	No

[a] For standard Tyler sieves.

Table III-6. Results of chemical analysis classified by men taking the samples

(Means of 12 samples in per cent)

Fertilizer and Element	Men 1	2	3	4	Range or Maximum Difference	Statistically Significant?
A. 10–10–10, granulated						
Nitrogen	9.90	9.96	9.94	9.89	0.07	No
Total phosphorus	10.90	10.92	10.83	10.80	0.12	At 0.01 level
Available phosphorus	10.76	10.82	10.72	10.71	0.11	At 0.001 level
Potash	10.68	10.74	10.57	10.63	0.17	No
B. 0–20–20, powdered						
Total phosphorus	20.98	21.02	20.92	21.12	0.20	No
Available phosphorus	20.49	20.53	20.43	20.64	0.21	No
Potash	20.02	20.14	20.15	20.05	0.13	No
C. 5–10–10, powdered						
Nitrogen	5.40	5.45	5.39	5.42	0.06	No
Total phosphorus	10.97	10.95	10.96	10.98	0.03	No
Available phosphorus	10.42	10.41	10.42	10.43	0.02	No
Potash	10.99	11.09	11.08	11.08	0.10	No
D. 8–16–16, granulated						
Nitrogen	8.25	8.15	8.24	8.26	0.11	No
Total phosphorus	17.32	17.31	17.24	17.20	0.12	No
Available phosphorus	16.89	16.89	16.83	16.77	0.12	No
Potash	16.43	16.45	16.53	16.45	0.10	No

the four men in another block of samples from the same fertilizer. In other words, the men were significantly inconsistent in their systematic effects. No significant interactions were found to exist between men and instruments.

The above suggests that a man may introduce a small systematic effect in the results obtained from sampling, but there is no evidence that these systematic effects vary with the instrument used.

4. Relative Systematic Effects of Instrument Sampling vs. Riffling

Section 2, above, was concerned with the systematic effects of the instruments relative to each other. This section deals with systematic effects of the instruments as a whole relative to another method of sampling.

In the experiment certain bags were sampled by instruments and the same bags were also sampled by riffling. In particular, three blocks of seven bags each were sampled by one of three instruments selected at random. Two samples were drawn from each bag and each block of samples was sent to a separate laboratory. From each of the seven bags in each block, two riffles were also taken and sent to the same laboratory as the instrument samples. The experiment thus provides information on the extent instrument samples are biased relative to riffles.

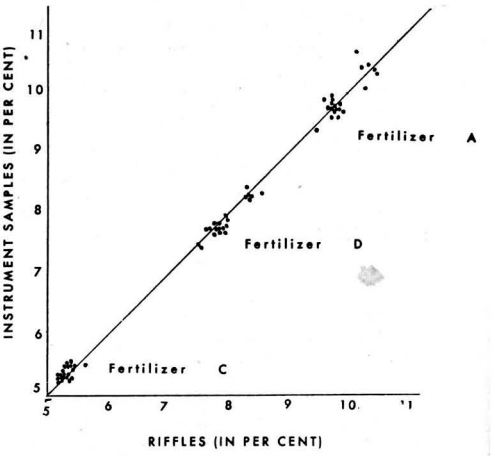


Fig. III-1—Comparison of instrument samples with riffles: Nitrogen.

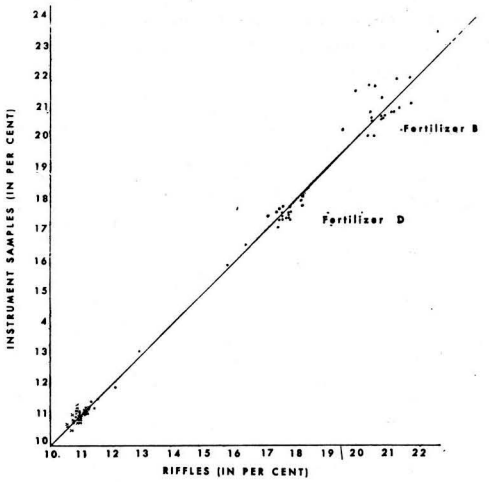


Fig. III-2—Comparison of instrument samples with riffles: Total phosphorus.
A dot on the lower part of the graph represents samples from Fertilizer A. An X on the lower part of the graph represents samples from Fertilizer C.

Table A 5 of the Appendix compares the mean of the two instrument samples and the mean of the two riffles from each bag for each type of fertilizer. The results are shown graphically in Figs. III-1, III-2, III-3, and III-4.

Insofar as the instrument samples and riffles match, a point on the chart will fall on a 45° line, through the origin. If a point falls above this 45° line, it indicates a higher percentage in the instrument samples; if a point falls below this line, it indicates a higher percentage in the riffles.

Table A 6 of the Appendix shows the analyses of variance for the data of Table A 5. No attempt is made in Table A 6 to study the variation between samples from the same bag; the primary purpose of the analysis was to determine significant differences between types of sampling, *viz.*, by instruments and by riffling. (*See,* however, Section 5 below and Chapter V.)

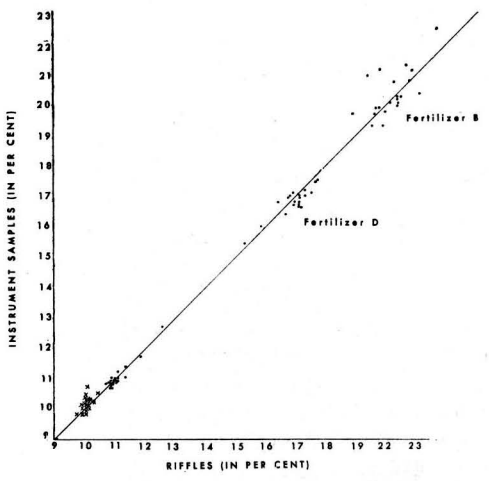


Fig. III-3—Comparison of instrument samples with riffles: Available phosphorus.
A dot on the lower part of the graph represents samples from Fertilizer A. An X on the lower part of the graph represents samples from Fertilizer C.

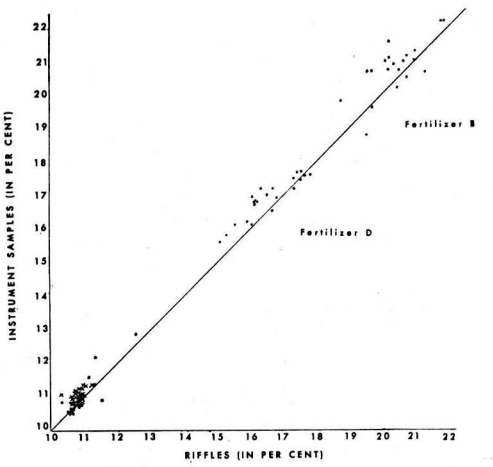


Fig. III-4—Comparison of instrument samples with riffles: Potash.
A dot on the lower part of the graph represents samples from Fertilizer A. An X on the lower part of the graph represents samples from Fertilizer C.

These tables and charts reveal the following:

Fertilizer A.—There are no significant mean differences between instrument sampling and riffling for any of the chemical elements.[7]

Fertilizer B.—The two types of sampling differ significantly with respect to their mean potash content. Fig. III-4 shows that riffles tend to contain lower percentages of potash than instrument samples of Fertilizer B. The mean difference is 0.39%.

[7] In Table A 7 of the Appendix, total and available phosphorus have significant interactions involving type of sampling when the second week's results are used in place of the first week's. This is due to the type of sampling having different mean effects in different blocks that cancel out over all blocks.

Table III-7. Means and ranges of absolute differences of two instrument samples and two riffles from the same bag. Fertilizer A

(Data in per cent)

Fertilizer and Chemical Element	Block[a]	Instrument Samples: First Week Mean	First Week Range	Second Week Mean	Second Week Range	Riffles Mean	Riffles Range	Grand Mean
A. 10–10–10, granulated								
N	1	.200	.40	.086	.20	.214	.40	
	2	.143	.34	.084	.13	.047	.08	
	3	.121	.25	.143	.25	.114	.30	
	Mean	.155		.104		.125		.128
						Equivalent standard deviation[b]		.113
TP	1	.114	.20	.071	.20	.057	.10	
	2	.050	.09	.194	.44	.070	.11	
	3	.093	.25	.086	.20	.043	.10	
	Mean	.086		.117		.057		.087
						Equivalent standard deviation[b]		.077
AP	1	.107	.20	.079	.20	.093	.20	
	2	.036	.02	.211	.37	.067	.09	
	3	.107	.30	.114	.25	.050	.10	
	Mean	.083		.135		.070		.096
						Equivalent standard deviation[b]		.085
K	1	.107	.25	.079	.05	.093	.30	
	2	.151	.23	.097	.40	.160	.14	
	3	.181	.26	.279	.58	.300	.68	
	Mean	.147		.151		.184		.161
						Equivalent standard deviation[b]		.143

[a] Tests of Blocks 1, 2, and 3 were run by Laboratories 1, 2, and 3 respectively, but the instruments and men used in taking the instrument samples varied at random from block to block.

[b] This is an unbiased estimate of the population standard deviation derived by dividing the grand mean difference by 1.128 (that is $E(R/1.128) = \sigma$ for $n = 2$). Cf. A. J. Duncan, *Quality Control and Industrial Statistics*, 2nd Ed., Richard D. Irwin, Inc., Homewood, Ill., 1959, p. 112.

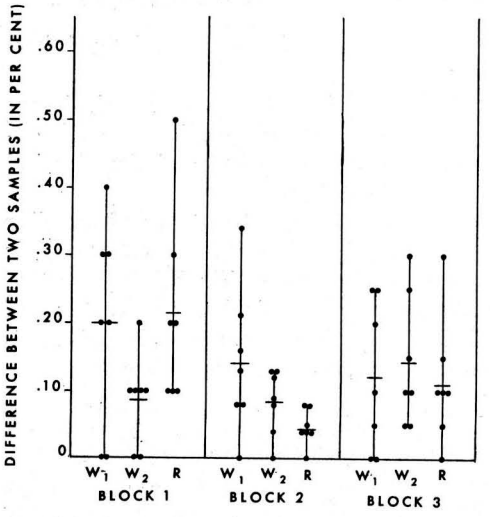


Fig. III-5—Comparison of variations in instrument samples with variations in riffles from the same bag: Fertilizer A, Nitrogen.

The two types of sampling also show significant interactions in the case of total phosphorus. This is true likewise for available phosphorus. It means that instrument sampling and riffling have different mean effects in different blocks.

Fertilizer C.—Mean differences between the two types of sampling are significant at the 0.05 level or better for nitrogen, available phosphorus, and potash and at the 0.10 level for total phosphorus. In every case the instrument samples show higher percentages of the chemical elements than do the riffles. It will be noted, however, that while the differences may be statistically significant, they are not absolutely large, the differences in favor of instrument samples being: total phosphorus, + 0.07%; available phosphorus, + 0.07%; nitrogen, + 0.07%; and potash, + 0.17%. The sta-

tistical significance arises from the low sampling and test errors with Fertilizer C. The reason why sampling by instruments should show a positive systematic effect relative to the results obtained by riffling *for all chemical elements* is not known. The results are simply reported for the record.

Fertilizer D.—There is a significant mean difference between the two types of sampling in respect to the per cent potash, the per cent potash being greater with instrument samples than with riffles. The mean difference is 0.39%.

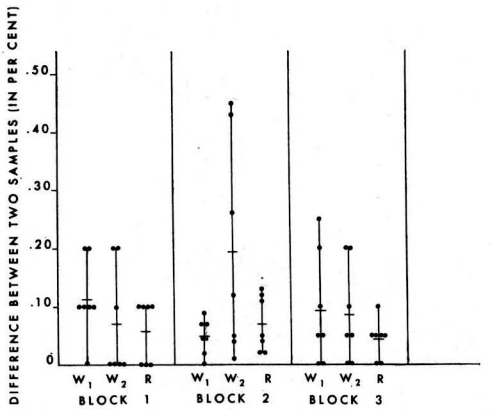


Fig. III-6—Comparison of variations in instrument samples with variations in riffles from the same bag: Fertilizer A, Total phosphorus.

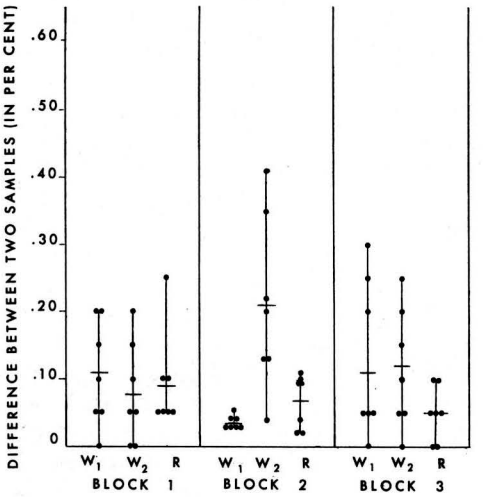


Fig. III-7—Comparison of variations in instrument samples with variations in riffles from the same bag: Fertilizer A, Available phosphorus.

Table III-8. Means and ranges of absolute differences of two instrument samples and two riffles from the same bag. Fertilizer B

(Data in per cent)

Fertilizer and Chemical Element	Block[a]	Instrument Samples: First Week Mean	First Week Range	Second Week Mean	Second Week Range	Riffles Mean	Riffles Range	Grand Mean
B. 0–20–20, powdered								
TP	1	.314	.70			.143	.30	
	2	.160	.30	.223	.32	.651	1.30	
	3	.314	.60	.264	.55	.293	.80	
	Mean	.263		.244		.362		.295
						Equivalent standard deviation[b]		.262
AP	1	.293	.60			.157	.40	
	2	.180	.18	.223	.32	.649	1.24	
	3	.321	.60	.286	.55	.314	.65	
	Mean	.265		.255		.373		.303
						Equivalent standard deviation[b]		.269
K	1	.329	.50			.157	.30	
	2	.726	.77			.886	1.47	
	3	.291	.48	.311	.46	.200	.54	
	Mean	.449		.311		.414		.414
						Equivalent standard deviation[b]		.367

[a] *See* footnote [a] to Table III-7. [b] *See* footnote [b] to Table III-7.

5. Relative Precision of Instrument Samples and Riffles

The data that were used to compare the systematic effects of instrument samples and riffles were means of two samples and two riffles from the same bag. Here we shall analyze the differences between the paired instrument samples and the differences between the paired riffles as a means of studying the precisions (i.e., the reproducibilities) of these two methods of sampling. The basic data for Fertilizer A are shown in Table III-7 and graphically in Figs. III-5, III-6, III-7, and III-8. A smaller amount of data for Fertilizers B, C, and D is presented in Tables III-8, III-9, and III-10. Table III-7 and Figs. III-5, III-6, III-7, and III-8 show in each case the absolute difference between two instrument samples and the absolute difference between two riffles from the same bag. For Fertilizer A two

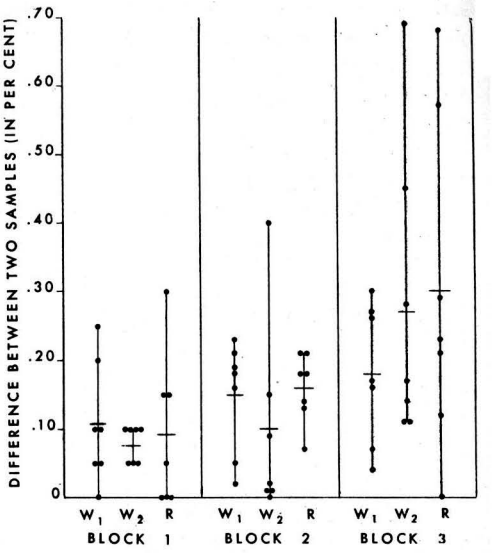


Fig. III-8—Comparison of variations in instrument samples with variations in riffles from the same bag: Fertilizer A, Potash.

Table III-9. Means and ranges of absolute differences of two instrument samples and two riffles from the same bag. Fertilizer C

(Data in per cent)

Fertilizer and Chemical Element	Block[a]	Instrument Samples: First Week Mean	First Week Range	Second Week Mean	Second Week Range	Riffles Mean	Riffles Range	Grand Mean
C. 5–10–10, powdered								
N	1	.086	.15	.093	.10	.171	.35	
	2	.133	.27			.074	.13	
	3	.029	.10	.057	.15	.029	.05	
	Mean	.082		.075		.091		.084
						Equivalent standard deviation[b]		.074
TP	1	.129	.40			.300	.60	
	2	.246	.59			.129	.20	
	3	.093	.15	.150	.35	.157	.15	
	Mean	.156		.150		.197		.173
						Equivalent standard deviation[b]		.153
AP	1	.129	.35			.293	.60	
	2	.200	.58			.117	.20	
	3	.093	.10	.150	.25	.150	.20	
	Mean	.140		.150		.187		.162
						Equivalent standard deviation[b]		.144
K	1	.150	.30			.179	.30	
	2	.199	.32			.130	.40	
	3	.199	.32	.094	.19	.123	.24	
	Mean	.182		.094		.144		.153
						Equivalent standard deviation[b]		.136

[a] *See* footnote [a] to Table III-7. [b] *See* footnote [b] to Table III-7.

Table III-10. Means and ranges of absolute differences of two instrument samples and two riffles from the same bag. Fertilizer D

(Data in per cent)

Fertilizer and Chemical Element	Block[a]	Instrument Samples: First Week Mean	First Week Range	Second Week Mean	Second Week Range	Riffles Mean	Riffles Range	Grand Mean
D. 8–16–16, granulated								
N	1	.300	.30			.171	.30	
	2	.161	.32			.090	.25	
	3	.107	.50			.143	.35	
	Mean	.189				.135		.163
						Equivalent standard deviation[b]		.144
TP	1	.100	.40			.100	.30	
	2	.280	.45			.237	.36	
	3	.164	.40			.229	.50	
	Mean	.181				.189		.185
						Equivalent standard deviation[b]		.164
AP	1	.114	.40			.107	.30	
	2	.290	.55			.194	.31	
	3	.129	.30			.193	.35	
	Mean	.178				.165		.172
						Equivalent standard deviation[b]		.152
K	1	.179	.50			.314	.45	
	2	.514	1.09			.264	.56	
	3	.167	.37			.197	.30	
	Mean	.287				.258		.273
						Equivalent standard deviation[b]		.242

[a] *See* footnote [a] to Table III-7. [b] *See* footnote [b] to Table III-7.

sets of chemical determinations were made a week apart. In Figs. III-5, III-6, III-7, and III-8, the two weeks are distinguished by W_1 and W_2.

In taking the instrument samples, the instruments x, y, and z together with the men who used them were assigned at random to the various "blocks" of tests so the riffling could be compared with an average of instrument sampling. It is not believed that the instruments or their use differed sufficiently to give rise to any important difference in the within-bag sampling variation. The riffling was done by the same riffle and in the same manner with all bags riffled. All tests in each block were run by the same laboratory.

A study of Tables III-7 to III-10 and Figs. III-5 to III-8 reveals that in no case was there a consistent difference over all three blocks between the mean sample-to-sample absolute difference for instrument samples and the mean sample-to-sample difference for riffles. The analyses of variance of the absolute differences for all fertilizers also fail to show a single significant mean difference between the precision of samples taken by instruments and that of samples taken by riffles. (*See* Note 10 of the Appendix.)

The results of the experiment suggest strongly, therefore, that for the fertilizers studied, the precision of instrument samples and riffles is the same.[8]

[8] Although the experiment thus indicates no difference in the mean precision of the two methods of sampling, a glance at Figs. III-5 to III-8, especially Fig. III-7, suggests a possible lack of consistency in precision. This is studied in detail in Note 11 of the Appendix and in Figs. A 1 to A 4 following Note 10. This more detailed analysis shows a definite "lack of control" in the variability of the absolute differences. That this is due, however, to any inconsistency in the precision of either of the two methods of sampling is difficult to believe. A more plausible conclusion is that the lack of control is due to inconsistency in laboratory procedures. For further discussion, *see* Note 11 of the Appendix.

CHAPTER IV

SYSTEMATIC VARIATIONS AND PRECISIONS OF CHEMICAL ANALYSES

1. Systematic Variations of the Laboratories

In one part of the experiment, instrument samples from the same bag were ground to pass a 35-mesh sieve, pooled, and subdivided by riffling into eight portions. Two of these portions were assigned at random to each of the three laboratories participating in the experiment. The whole was replicated three times, yielding a total of 6 tests for each element by each laboratory. These tests provide a basis for interlaboratory comparisons.

The means of the three laboratories for the various chemical elements and fertilizers are presented in Table IV-1. It will be noticed that the differences between the laboratories are sizeable. In all but two cases, the range of the laboratory means for a given chemical element is at least 0.10 and in two cases runs as high as 0.50. In most cases these interlaboratory mean differences are several times the instrumental mean differences of Table III-4.

Not only are the differences between the laboratory means sizeable but they are in

Table IV-1. Mean determinations of chemical content for six samples from three bags classified by laboratories

(Data in per cent. First week only)

Fertilizer	Lab. 1	Lab. 2	Lab. 3	Range or Maximum Difference	Statistically Significant?
Nitrogen					
A. 10–10–10, granulated	10.28	9.70	9.78	0.58	At 0.01 level
C. 5–10–10, powdered	5.58	5.38	5.37	0.21	At 0.05 level
D. 8–16–16, granulated	8.22	7.72	7.72	0.50	At 0.001 level
Total Phosphorus					
A. 10–10–10, granulated	11.40	11.17	11.05	0.35	No[a]
B. 0–20–20, powdered	21.62	21.48	21.25	0.37	No
C. 5–10–10, powdered	11.48	11.22	11.20	0.28	At 0.01 level
D. 8–16–16, granulated	17.67	17.73	17.57	0.16	No
Insoluble Phosphorus					
A. 10–10–10, granulated	0.21	0.18	0.26	0.08	No
B. 0–20–20, powdered	0.73	0.48	0.80	0.32	At 0.05 level
C. 5–10–10, powdered	0.83	0.66	0.90	0.24	No[a]
D. 8–16–16, granulated	0.63	0.50	0.58	0.13	No[a]
Potash					
A. 10–10–10, granulated	10.80	10.82	10.85	0.05	No[a]
B. 0–20–20, powdered	20.32	20.37	20.43	0.11	No
C. 5–10–10, powdered	11.70	11.42	11.52	0.28	No[a]
D. 8–16–16, granulated	16.77	16.60	16.85	0.25	No

[a] But the laboratories do differ significantly from bag to bag. *See* Note 12 of the Appendix.

most cases statistically significant. The analysis of variance of the results is shown in Table A 9 in the Appendix. For the A, C, and D Fertilizers either the laboratory main effects or the laboratory-bag interactions are significant at the 0.05 level for all chemical elements with the exception of insoluble phosphorus in Fertilizer A and total phosphorus and potash in Fertilizer D, and even the latter are close to the 0.05 point. For Fertilizer B there are no significant laboratory main effects or interactions except those for insoluble phosphorus. This is not because the variations in main effects and interactions are small, but because the error variations are generally higher in this case than with other fertilizers. (*See* Appendix, Note 12.)

The significant laboratory main effects suggest the existence of clear-cut systematic variations between laboratories, say, systematic effects due to differences in analytical methods or differences in the personal biases of laboratory technicians. Table IV-1 suggests that such systematic effects exist for nitrogen for Fertilizer A, insoluble phosphorus for Fertilizer B, total phosphorus and nitrogen for Fertilizer C, and nitrogen for Fertilizer D.

The significant laboratory-bag interactions suggest that the laboratories have systematic variations relative to each other, but that they are not consistent in these systematic variations. Since the two samples from a given bag were usually tested by each laboratory on the same day, the significant laboratory-bag interactions may simply be evidence of a day-to-day effect rather than a systematic laboratory effect. The analysis of the next sections shows that day-to-day effects do appear within the same laboratory and hence would also appear in interlaboratory comparisons when not eliminated by the design.

2. Intra-Laboratory Variations

Day-to-Day Variations as Estimated from Two Days a Week Apart

The original experiment called for all three laboratories to run two tests on a number of samples a week apart. Since other experiments had reported on the variation between "duplicates," it was believed that a study of week-to-week variation might yield new information on intra-laboratory variations. Unfortunately, as noted in Chapter I, there was a misunderstanding at the time the original experiment was planned as to the meaning of the word "duplicate" as employed in the earlier experiments. In the language of the statistician the word "duplicate" refers to a second test run under essentially the same conditions as the first. The "variation between duplicates" reported in some earlier experiments was variation between tests run on different days and thus contained the day-to-day variation as well as variation within days.

A supplementary experiment was therefore run to secure separate measures of these two types of intra-laboratory variations. In this section we shall discuss estimation of the day-to-day variations as reflected in differences between two days a week apart. The results of the supplementary experiment are discussed in the next section.

Tests a week apart were provided for instrument samples only and primarily for tests on Fertilizer A. Provision was also made for a limited number of tests a week apart on some of the elements of Fertilizers B and C. (*See* Appendix, Note 3.) Although not called for in the experiment as planned, Laboratory 3 ran tests a week apart on all samples of Fertilizers B and C tested in this part of the experiment. Laboratory 2, following the directions of the experiment, ran tests a week apart for phosphorus only in Fertilizer B and Laboratory 1 ran tests a week apart for nitrogen only in Fertilizer C.

Fortunately for the subsequent analysis of the results, practically all samples run in a given week were run on the same day. The exceptions occurred only in the case of Fertilizer A and because of this the analysis of intra-laboratory variations for this fertilizer had to be based on a smaller amount of data than would otherwise have been the case.

Figure IV-1 shows the day-to-day differences for total phosphorus of Fertilizer A

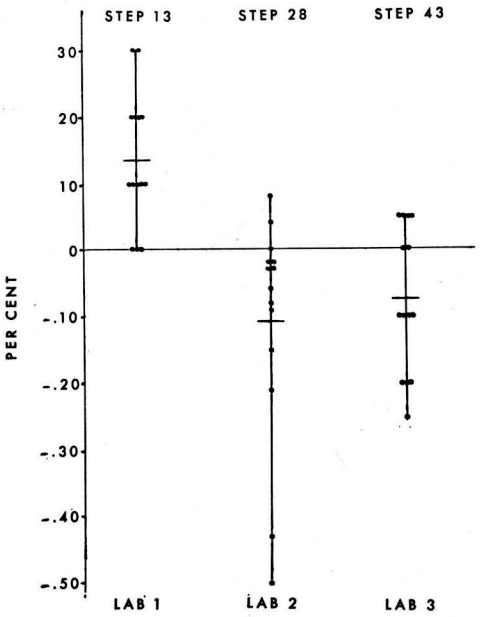


Fig. IV-1—Differences between tests a week apart: Fertilizer A, Total phosphorus (P_2O_5).

as tested by each laboratory when the two days are a week apart and Fig. IV-2 shows the day-to-day differences for nitrogen of Fertilizer C. The means of the day-to-day differences for homogeneous sets of sample data for all chemical elements of Fertilizers A and C are presented in Table IV-2, together with 0.95 confidence limits for the "true" or universe means. If the confidence limits reported for the "universe means" are both positive or both negative, it indicates the likely existence of a real day-to-day variation, the limits suggesting the range within which this variation actually lies. If the confidence limits bracket zero, we conclude (until subsequent data possibly show otherwise) that there is no real day-to-day variation.

On the above basis, Table IV-2 indicates the existence of a real day-to-day variation within the same laboratory for:

(1) total phosphorus, insoluble phosphorus, available phosphorus, and nitrogen in Fertilizer A, with potash a borderline case for one laboratory; and

(2) nitrogen for Fertilizer C.

Day-to-day variations are strikingly missing in the case of Fertilizer B and the reason for this is obscure. No tests a week apart were run on Fertilizer D, so no results can be reported for this fertilizer.

It will be noted from Table IV-2 that the significant day-to-day variations were in the neighborhood of 0.10% to 0.15%. If we multiply by a factor[9] of 1.5 to allow for the fact that the differences in Table IV-2 are in reality ranges of two items while those shown in Tables III-4 and IV-1 are ranges of three items, it will be noted that these day-to-day variations are distinctly less than the overall laboratory systematic effects noted in Section 1 above, but of about the same order of magnitude as the instrumental mean differences of Table III-4.

In Note 13 of the Appendix, it is shown how the estimates of day-to-day differences given in the first column of Table IV-2 can be converted into estimates of the net standard deviation of day-to-day variations. The results of this conversion are shown in the third column of Table IV-2 for those day-to-day variations that are shown in the second column to be statistically significant. Since the estimates of the net standard deviations are in each case based on a difference between a single pair of means, no attempt has been made to compute confidence limits for universe values. The results are merely useful for comparison with those obtained

[9] *cf.* A. J. Duncan, *Quality Control and Industrial Statistics*, Rev. Ed., Table D1, p. 872. The ratio 1.693/1.128 = 1.5.

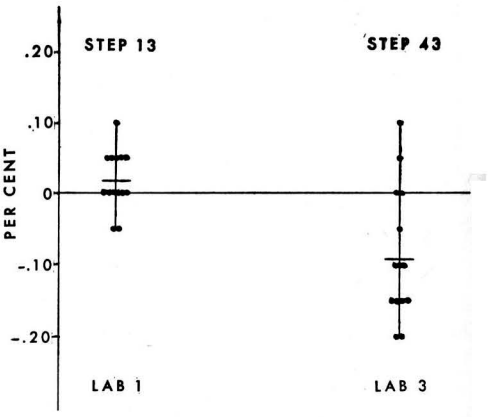


Fig. IV-2—Differences between tests a week apart: Fertilizer C, Nitrogen.

Table IV-2. Estimation of day-to-day variations from two days a week apart

(Means of day-to-day differences, in per cent)

Chemical Element and Laboratory	Day-to-Day Variation			Degrees of Freedom for Random Error[c]
	Estimate of Day-to-Day Difference[a]	0.95 Confidence Limits for Day-to-Day Difference	Estimated Net Standard Deviation of Day-to-Day Variation[b]	
A. 10–10–10, Granulated				
Nitrogen				
Lab. 1	0.14	0.03 to 0.20	0.076	35
Lab. 2(a)[d]	−0.01	−0.11 to 0.09	—	35
(b)[d]	−0.07	−0.21 to 0.06	—	35
Lab. 3	0.05	−0.04 to 0.13	—	35
Total phosphorus				
Lab. 1	0.14	0.07 to 0.21	0.093	39
Lab. 2	−0.11	−0.18 to −0.04	0.072	39
Lab. 3	−0.08	−0.15 to −0.00	0.047	39
Insoluble phosphorus				
Lab. 1	−0.01	−0.03 to 0.01	—	38
Lab. 2	0	−0.03 to 0.03	—	38
Lab. 3	−0.06	−0.08 to −0.04	0.042	38
Available phosphorus				
Lab. 1	0.13	0.05 to 0.20	0.088	38
Lab. 2	−0.11	−0.19 to −0.03	0.070	38
Lab. 3	−0.02	−0.09 to 0.06	—	38
Potash				
Lab. 1	−0.02	−0.08 to 0.04	—	26
Lab. 2	−0.06	−0.12 to 0.00	—	26
Lab. 3	0.12	−0.05 to 0.29	—	13
B. 0–20–20, Powdered				
Total phosphorus				
Lab. 2	−0.02	−0.16 to 0.13	—	26
Lab. 3	0.01	−0.14 to 0.16	—	26
Available phosphorus				
Lab. 2	−0.03	−0.18 to 0.13	—	26
Lab. 3	−0.00	−0.16 to 0.15	—	26
Potash				
Lab. 3	−0.08	−0.29 to 0.13	—	13
C. 5–10–10, Granulated				
Nitrogen				
Lab. 1	0.02	−0.02 to 0.06	—	26
Lab. 2	−0.09	−0.13 to −0.05	.059	26

(Continued)

Table IV-2 (*Continued*)

(Means of day-to-day differences, in per cent)

Chemical Element and Laboratory	Day-to-Day Variation: Estimate of Day-to-Day Difference[a]	Day-to-Day Variation: 0.95 Confidence Limits for Day-to-Day Difference	Day-to-Day Variation: Estimated Net Standard Deviation of Day-to-Day Variation[b]	Degrees of Freedom for Random Error[c]
C. 5-10-5, Granulated—(*Continued*)				
Total phosphorus				
Lab. 3	0.11	−0.00 to 0.22	—	13
Insoluble phosphorus				
Lab. 3	0.03	−0 to 0.06	—	13
Available phosphorus				
Lab. 3	0.08	−0.04 to 0.20	—	13
Potash				
Lab. 3	0.03	−0.05 to 0.11	—	13

[a] Rounded off to two decimals.
[b] *See* Note 13 of Appendix.
[c] In most cases the within-day variation was pooled for all laboratories to get a larger base for estimating the random error.
[d] Lab. 2 ran some tests one day and some another.

Table IV-3. Estimates of the standard deviations of day-to-day variation

Fertilizer and Chemical Element	Mean Square: Between Days Within Labs.	Mean Square: Test Error	Standard Deviation of Day-to-Day Variation: Estimate	Standard Deviation of Day-to-Day Variation: Estimated 0.95 Confidence Limits
A. 10-10-10, granulated				
AP	0.0138	0.0055	0.06	0.01–0.13
N	0.0365	0.0100	0.12	0.05–0.21
K	0.0126	0.0039	0.07	0.02–0.12
B. 0-20-20, powdered				
AP	0.1387	0.0130	0.25	0.15–0.42
K	0.0300	0.0155	0.09	0–0.19

in the next section from analysis of the data yielded by the supplementary experiment.

Day-to-Day Variations as Estimated from the Supplementary Experiment

In the supplementary experiment the three laboratories each ran duplicate tests on the same sample (but different from laboratory to laboratory) on seven different days. The standard deviation of the variation from day to day will thus give some indication of the average size of this variation. Measurement of this is attempted in Table IV-3. Here are presented estimates of the standard deviation of day-to-day variation together with 0.95 confidence limits for these estimates.[10] (*See* Appendix, Note 14, for method of computation.)

The results presented in Table IV-3 agree reasonably well with those presented in Table IV-2 for Fertilizer A. For Fertilizer B, however, Table IV-3 shows significantly large standard deviations for day-to-day variation whereas Table IV-2 shows practically no day-to-day variation for this fertilizer.

[10] Results are available only for Fertilizers A and B. The laboratories also reported only on *available* phosphorus.

Table IV-4. Estimates of the standard deviations of within-day variation from tests on two days a week apart

Chemical Element and Laboratory	Standard Deviation of Within-Day Variation	
	Estimate (%)	0.95 Confidence Limits (%)
A. 10–10–10, granulated		
Nitrogen		
Lab. 1	0.12	0.09 to 0.20
Lab. 2(a)	0.07	0.05 to 0.14
(b)	0.11	0.07 to 0.31
Lab. 3	0.10	0.07 to 0.18
Pooled Labs.	0.10	0.08 to 0.14
Total phosphorus		
Lab. 1	0.07	0.05 to 0.11
Lab. 2	0.12	0.09 to 0.19
Lab. 3	0.08	0.06 to 0.12
Pooled Labs.	0.09	0.08 to 0.12
Insoluble phosphorus		
Lab. 1	0.03	0.02 to 0.04
Lab. 2	0.04	0.03 to 0.06
Lab. 3	0.03	0.02 to 0.05
Pooled Labs.	0.03	0.03 to 0.04
Available phosphorus		
Lab. 1	0.06	0.04 to 0.09
Lab. 2	0.12	0.08 to 0.19
Lab. 3	0.08	0.06 to 0.13
Pooled Labs.	0.10	0.08 to 0.13
Potash		
Lab. 1	0.07	0.05 to 0.11
Lab. 2	0.09	0.07 to 0.15
Lab. 3	0.21	0.15 to 0.34
Pooled Labs. 1 and 2	0.08	0.06 to 0.11
B. 0–20–20, powdered		
Total phosphorus		
Lab. 2	0.19	0.14 to 0.31
Lab. 3	0.18	0.13 to 0.29
Pooled Labs.	0.19	0.15 to 0.26
Insoluble phosphorus		
Lab. 2	0.05	0.04 to 0.08
Lab. 3	0.09	0.06 to 0.14
Pooled Labs.	0.07	0.06 to 0.10
Available phosphorus		
Lab. 2	0.18	0.13 to 0.29
Lab. 3	0.21	0.16 to 0.34
Pooled Labs.	0.20	0.16 to 0.27
Potash		
Lab. 3	0.26	0.19 to 0.43
C. 5–10–10, granulated		
Nitrogen		
Lab. 1	0.03	0.02 to 0.05
Lab. 3	0.07	0.05 to 0.11
Total phosphorus		
Lab. 3	0.13	0.10 to 0.22
Insoluble phosphorus		
Lab. 3	0.04	0.03 to 0.07
Available phosphorus		
Lab. 3	0.14	0.10 to 0.23
Potash		
Lab. 3	0.10	0.07 to 0.16

Since the results presented in Table IV-2 are for a difference of two days only, their similarity may well be due to chance. Nevertheless, the fact that this similarity in day effects should hold for two different pairs of days, and for two different chemical elements, raises some doubt that it is due to chance. Of the two sets of data, those of Table IV-3 are more reliable estimates of the true day-to-day standard deviations than those of Table IV-2 since they are based on a larger number of day-to-day differences.

Within-Day Laboratory Variations

The variations in results of tests run on the same material on the same day by a given laboratory may be termed "within-day" variations. It might be possible to subdivide these variations further into "between-batch" and "within-batch" variations if the experiment had been designed to do this. As it stands, it is not known whether a given set of tests reported run on a given day were run in a single batch or in several batches. If there is a batch effect and tests on one day were run in more than one batch, what is reported here as within-day variation will be a composite of between-batch and within-batch variation. In the supplementary experiment in which it was

requested that two duplicate tests be run on the same day, it is likely that these were in each case run in the same "batch." In the sets of tests run a week apart, as called for in the original experiment, there is a somewhat smaller but still high probability that the seven tests of each set were run in the same batch. On the other hand, if all tests in a single day were also run in a single batch, then the between-batch variation is confounded with the day-to-day variation and the within-day variation becomes essentially within-batch variation. It is thus likely that the day-to-day variation discussed in the previous section contains whatever batch-to-batch variation that exists, and that the within-day variation discussed here is primarily within-batch variation. Further experimentation is needed to determine whether there are real batch-to-batch variations within a given day.

As noted in Section 2, above, "Day-to-Day Variations as Estimated from the Supplementary Experiment," the day-to-day differences in tests run on two days a week apart do not generally average to zero as might be expected but contain an overall day-to-day effect that yields a net positive or negative mean effect. If, however, this mean day-to-day effect is eliminated, it is believed that the residuals can be viewed as measuring within-day variation. Table IV-4 presents estimates of the standard deviations of these net within-day variations calculated from the day-to-day differences such as are shown in Figs. IV-1 and IV-2 together with 0.95 confidence limits for the "true" values.

Differences between duplicate tests run the same day are a direct measure of within-day variations. Estimates of the standard deviations of within-day variations computed from the differences between the duplicate tests yielded by the supplementary experiment are presented in Table IV-5.

It will be noted that for the various fertilizers and chemical elements for which

Table IV-5. Estimates of the standard deviations of within-day variation from duplicates run the same day

	Fertilizer and Chemical Element[a]	Standard Deviation of Test Error: Estimate (%)	Standard Deviation of Test Error: 0.95 Confidence Limits (%)
A.	10–10–10, granulated		
	AP	0.07	0.06–0.11
	N	0.10	0.08–0.14
	K	0.06	0.05–0.09
B.	0–20–20, powdered		
	AP	0.11	0.09–0.16
	K	0.13	0.10–0.18

[a] Measurement of TP was not undertaken for these samples.

Tables IV-4 and IV-5 are comparable, the results of the two methods of estimating the within-day variation agree quite well, with two exceptions. Except for Fertilizer B, the confidence intervals obtained by the two methods of estimation overlap considerably and the single estimates agree within 0.02–0.03 points. In the case of potash of Fertilizer B, however, there is a significant difference in the results obtained by the two methods. The 0.95 confidence limits for the within-day variation estimated from day-to-day differences a week apart are 0.19 to 0.43, whereas the 0.95 confidence limits for within-day variation measured directly from duplicates are 0.10–0.18. A similar situation occurs in comparison of the two measures of within-day variation of available phosphorus of Fertilizer B (*see* Tables IV-4 and IV-5). What the differences may be attributed to is unknown. It is possible that the larger variation includes some between-batch variation within a given day.

In general, the within-day variation would appear to be somewhat higher than the net between-day variation, but the differences are not very large—at most about 0.05 points. Unfortunately, the data do not permit widespread comparison.

CHAPTER V
VARIATIONS IN MANUFACTURE

1. Bag-to-Bag Variations

As noted in Chapter I, the bags of fertilizer used in the experiment were selected at random from the manufacturer's lot as it was bagged.

Figures V-1 to V-4 give examples of the bag-to-bag variation disclosed by the experiment, after adjustment has been made for the systematic laboratory effects noted in Chapter IV. Actually the data shown in these charts are deviations in per cent from the means of the bags tested by each laboratory. The original data were means of two riffles from the specified bags. Original data for all chemical elements and all fertilizers are given in Table A 10 in the Appendix.

For purposes of analysis, charts like Figs. V-1 to V-4 were constructed for all chemical elements of all fertilizers. In drawing these charts it was intended first of all to see if the process was in each case "in control" in the sense that the (adjusted) variations could be reasonably explained by a homogeneous set of chance forces. To do this, the bag-to-bag differences for each day were computed and averaged over all days to yield an estimate of variability as free as possible from the effects of "assignable causes." Three-sigma control limits based on these estimates of variability were plotted on the charts. (These limits were not drawn on Fig. V-4 because of the apparent lack of homogeneity.) It was then noted whether points fell outside the "control limits" or showed other evidence of non-random variation.

The second purpose of the "control chart" analysis was to make some estimate of the bag-to-bag variation that might be attained if "assignable causes" of systematic variation could be eliminated from the manufacturing process. For each chemical element of each fertilizer standard deviations were computed of the adjusted deviations such as shown in Figs. V-1 to V-4. When a chart

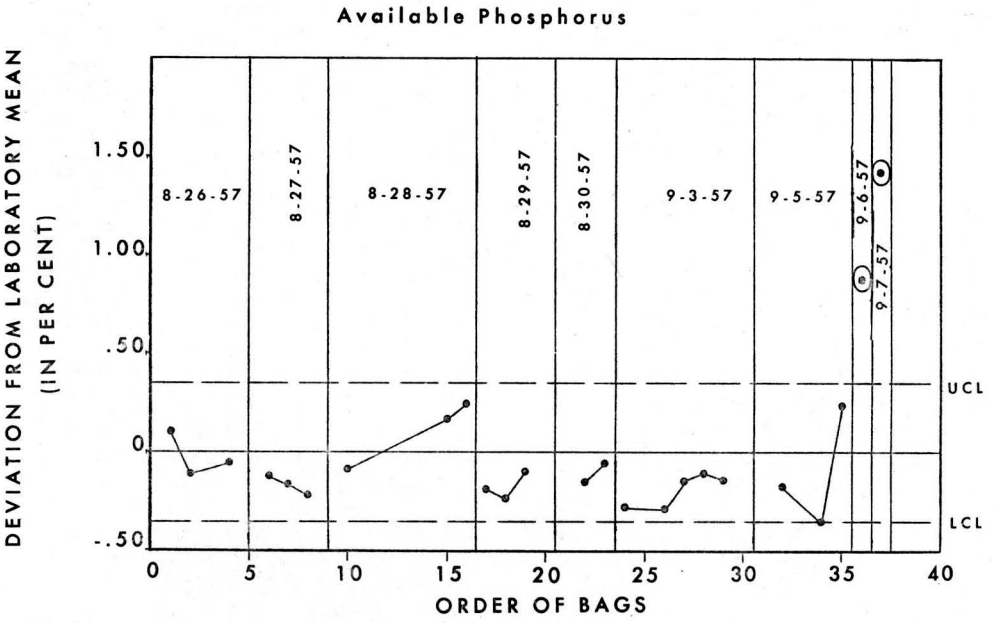


Fig. V-1—Bag-to-bag variations (adjusted for systematic laboratory effects): Fertilizer A. Upper and lower 3-sigma control limits indicated.

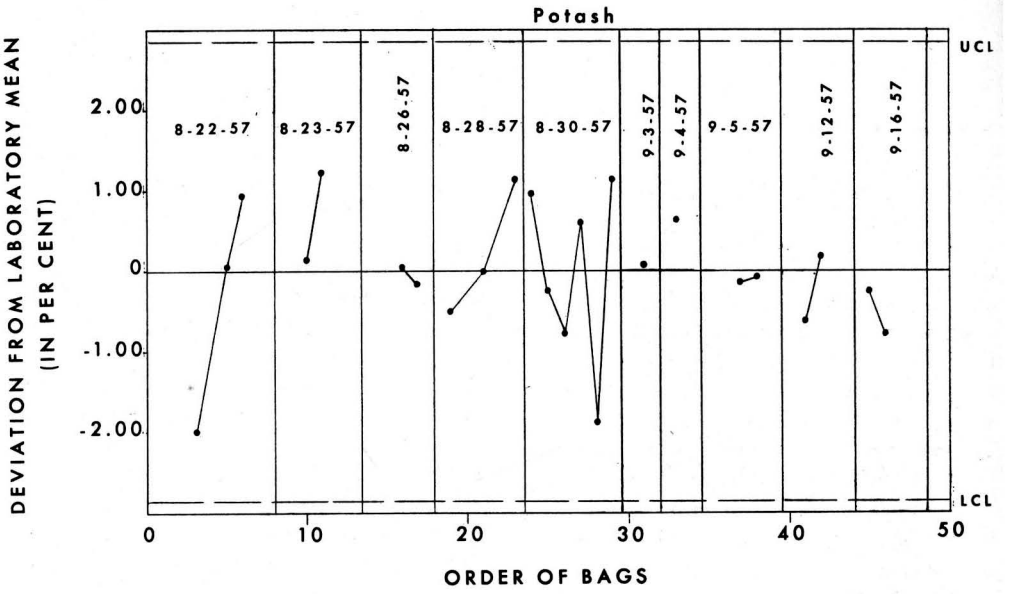


Fig. V-2—Bag-to-bag variations (adjusted for systematic laboratory effects): Fertilizer B. Upper and lower 3-sigma control limits indicated.

showed that a process was "not in control" because one or more points fell outside the "control limits," standard deviations were also computed for points only within "control limits." It is suggested that this second set of standard deviations are what the manufacturer might attain if he could eliminate the "assignable causes" of extreme variation.

The results obtained by the above analysis may be summarized as follows: It is to be noted that the standard deviations listed are standard deviations of *gross* bag-to-bag variation; i.e., bag-to-bag variation plus

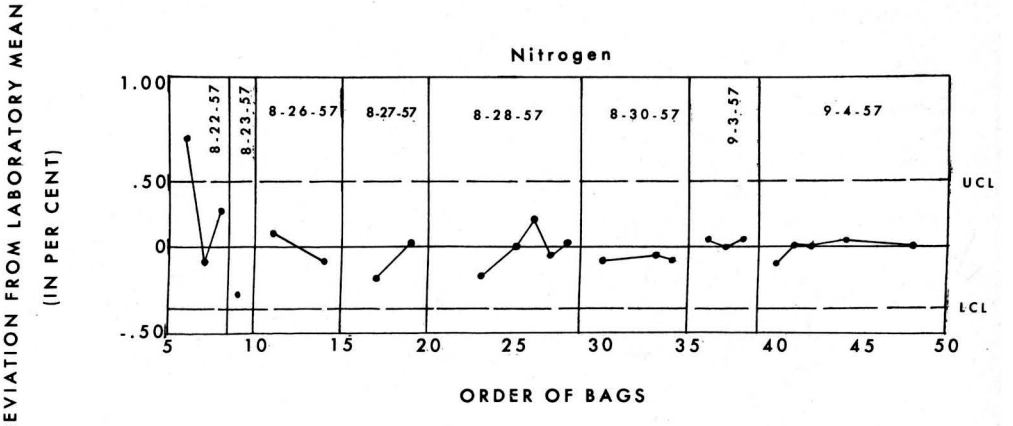


Fig. V-3—Bag-to-bag variation (adjusted for systematic laboratory effects): Fertilizer C. Upper and lower 3-sigma control limits indicated.

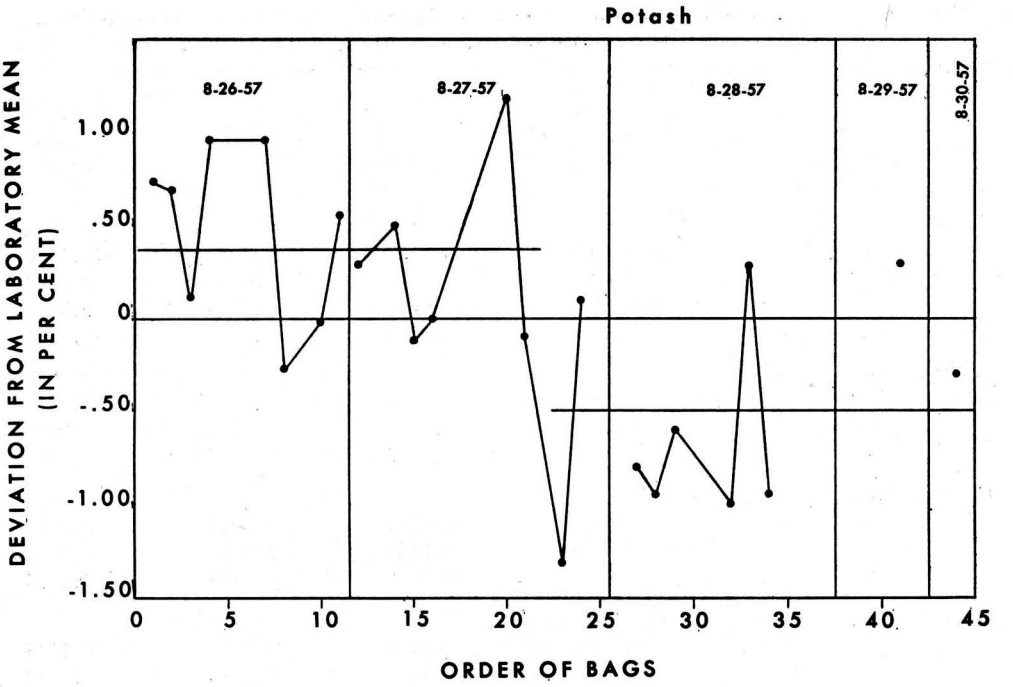


Fig. V-4—Bag-to-bag variations (adjusted for systematic laboratory effects): Fertilizer D.

within-bag sampling variation and test error (excluding systematic laboratory effects). Attempts at measuring net bag-to-bag variation are undertaken in the next chapter.

Fertilizer A.—The last two bags sampled from the bin were definitely out of line in their phosphorus content with respect to the other bags of the lot. (Compare Fig. V-1.) The potash content of the last bag was also far out of the "control limits." The bag-to-bag variation would thus have been much more uniform if the last two bags had not been included. The standard deviations of gross bag-to-bag variation are:

	(1) *All Bags*	(2) *Excluding Last 2 Bags*
N	0.14	0.14
TP	0.43	0.22
AP	0.39	0.19
K	0.45	0.31

Fertilizer B.—The bag-to-bag variations in Fertilizer B are large but appear to be "in control." (Compare Fig. V-2.) This is true for both phosphorus and potash. The standard deviations of gross bag-to-bag variations are:

TP	0.67
AP	0.66
K	0.85

Fertilizer C.—There is evidence here that the early bags in the lot were out of line with the others. This is shown for nitrogen in Fig. V-3. It was also true for potash. "Out of control" points for phosphorus appeared at more intermediate points. Whether these "out of control" variations were due to segregation in the bin or some other cause is not known. The standard deviations of gross-bag-to-bag variation are:

	(1) *All Bags*	(2) *Excluding First 4 Bags*
N	0.18	0.08
TP	0.25	0.19
AP	0.22	0.13
K	0.21	0.13

Fertilizer D.—Fertilizer D was made in two batches which were piled one on top of the other and it seems likely *a priori* that this would cause segregation. The statistical evidence shows that the later bags do tend to run high in phosphorus and low in

Table V-1. Comparison of absolute differences between two samples from the same bag taken (1) by a sampling instrument and (2) by riffling[a]

Fertilizer and Element	Instrument Sampling[a]		Riffling	
	Mean Absolute Difference (%)	Equivalent as a Standard Deviation (%)	Mean Absolute Difference (%)	Equivalent as a Standard Deviation (%)
A. 10–10–10, granulated				
N	0.1548	0.1372	0.1633	0.1448
TP	0.0857	0.0762	0.0567	0.0503
AP	0.0833	0.0736	0.0700	0.0621
K	0.1466	0.1300	0.1843	0.1634
B. 0–20–20, powdered				
TP	0.2629	0.2331	0.3624	0.3213
AP	0.2648	0.2347	0.3733	0.3309
K	0.4486	0.3977	0.3524	0.3124
C. 5–10–10, powdered				
N	0.0824	0.0730	0.0914	0.0810
TP	0.1557	0.1380	0.1967	0.1744
K	0.1824	0.1617	0.1438	0.1275
D. 8–16–16, granulated				
N	0.1895	0.1680	0.1348	0.1195
TP	0.1814	0.1608	0.1886	0.1672
AP	0.1776	0.1574	0.1648	0.1461
K	0.2914	0.2583	0.2586	0.2292

[a] Includes first week instrument samples only.

potash. (Compare Fig. V-4.) Unfortunately, however, these tendencies are the exact opposite of the results reported by the commercial laboratory for the two batches, which greatly weakens the argument that the segregation revealed in Fig. V-4 is due to the difference in batches.

The gross standard deviations for Fertilizer D are as follows:

	(1)All Bags	*(2) Selected Bags*
N	0.16	0.15 first 14 bags
TP	0.50	0.35 first 2 days only
AP	0.48	0.33 first 2 days only
K	0.68	0.61 first 14 bags

2. Variation Within Bags

It does not seem appropriate here to enter upon an academic discussion of what is meant by variation within bags or the standard deviation of within-bag variation. In this report we shall define within-bag variation as the variation in results that occurs when more than one sample is taken from a bag by the same method.

The experiment was conducted so that for a designated number of bags two samples were taken from the same bag by a given instrument, one sample from the left hand corner of the bag, the other from the right hand corner. The remainder of the bag was then riffled twice so as to yield two riffles of about 5 lbs each. The instrument samples were ground to a powder before reduction for chemical analysis and the riffle sample was also ground *in toto* by Laboratory 1 before reduction. Laboratories 2 and 3 reduced the 5 lb riffles by further riffling before grinding to a powder. In this section we shall discuss the differences between the two instrument samples and the differences between the two riffles as measures of gross within-bag variation. These differences are measures of *gross* within-bag variation because they also include laboratory test error.

Table V-1 presents, for 7 bags, the mean of absolute differences between samples from the same bag, for each element in each grade of fertilizer. Mean differences are presented

for both instrument samples and riffles. Since a difference is a range of a sample of two, it is possible to convert the mean difference in each case into an estimate of the gross within-bag standard deviation by simply dividing the mean difference by the so-called d_2 factor for a range of two, which is 1.128.

Table V-1 shows that the standard deviation of gross within-bag variation varies considerably for different fertilizers and different elements. For example, the standard deviation of gross within-bag variation for available phosphorus in Fertilizer A is about roughly one-half that of Fertilizer D. Again the standard deviation of the within-bag variation for nitrogen for Fertilizer A is roughly double that of Fertilizer C which means that the coefficients of variation are about the same since the nominal level of A is double that of C.

It should be noted once again that the squares of the within-bag standard deviations given in Table V-1 are in reality sums of two components of variance, the pure or net within-bag sample variance, and the laboratory test variance. The determination of the net within-bag sample variances of Fertilizers A, B, and C is discussed in Chapter VI.

CHAPTER VI

COMPONENTS OF VARIANCE AND TOLERANCES

1. Summary of Components of Variance

This section will summarize results pertaining to the various components of variance.

A unified comprehensive analysis is provided for Fertilizers A, B, and C by the results of Steps 13, 28, and 43 of the experiment[11] in which "block" (mostly laboratory) effects, day-to-day effects, bag-to-bag variations, sample-to-sample variations within bags, and elementary laboratory test errors are all involved. The estimated net standard deviations provided by these data for Fertilizers A, B, and C are given in Tables VI-1 and VI-2 and a comparison with results yielded in other parts of the experiment is offered in Table VI-3 together with some related data for Fertilizer D.

In studying Tables VI-1 and VI-2, the reader who is unacquainted with statistical analysis should note that confidence limits for net standard deviations may be highly unsymmetrical with respect to the sample estimate from which they are derived. For example, in Table VI-1 the 0.95 confidence limits for the standard deviation of the day-to-day variation within the same laboratory[12] for nitrogen of Fertilizer A are given as 0 to 0.35%, although the best single estimate of this standard deviation is given as 0.04%. Generally the best single estimate of the net standard deviation falls much closer to the lower confidence limit than the upper. This should be especially noted when studying Table VI-3. Confidence limits based on a very small number of degrees of freedom (effective sample size) are extremely

[11] *See* Note 3 of the Appendix.

[12] The table uses the technical expression "days within blocks." The major difference between blocks is the difference in the laboratories that ran the tests.

Table VI-1. Estimates of net standard deviations based on instrument samples

Source of Variation	Estimated Net Standard Deviation (%)	Estimated 0.95 Confidence Limits (%)
Fertilizer *A*: 10–10–10, Granulated		
Nitrogen[a]		
Blocks	0.31	0–10.43
Days *w.* Blocks	0.04	0–0.35
Bags *w.* Blocks	0.29	0.13–0.61
Samples *w.* Bags	0.04	0–0.14
Bags × Days *w.* Blocks	0.05	0–0.17
Residual	0.11	0.08–0.19
Total Phosphorus (P_2O_5)		
Blocks	0	0–0.39
Days *w.* Blocks	0.07	0.03–0.28
Bags *w.* Blocks	0.51	0.32–0.82
Samples *w.* Bags	0.04	0–0.10
Bags × Days *w.* Blocks	0	0–0.07
Residual	0.09	0.07–0.13
Available Phosphorus[a] (P_2O_5)		
Blocks	0	0–0.61
Days *w.* Blocks	0.06	0.02–0.25
Bags *w.* Blocks	0.50	0.30–0.85
Samples *w.* Bags	0.06	0–0.11
Bags × Days *w.* Blocks	0.02	0–0.08
Residual	0.08	0.06–0.12
Potash		
Blocks	0	0–0.97
Days *w.* Blocks	0	0–0.10
Bags *w.* Blocks	0.57	0.35–0.92
Samples *w.* Bags	0.08	0–0.16
Bags × Days *w.* Blocks	0.10	0–0.19
Residual	0.12	0.09–0.17
Fertilizer *B*: 0–20–20, Powdered		
Total Phosphorus (P_2O_5)		
Blocks[b]	0	0–1.51
Days *w.* Blocks	0	0–0.01
Bags *w.* Blocks	0.91	0.50–1.65
Samples *w.* Bags	0.16	0.05–0.31
Bags × Days *w.* Blocks	0.14	0.02–0.29
Residual	0.13	0.10–0.21
Available Phosphorus (P_2O_5)		
Blocks[b]	0	0–7.36
Days *w.* Blocks	0	0–0.05
Bags *w.* Blocks	0.85	0.47–1.54
Samples *w.* Bags	0.15	0–0.29
Bags × Days *w.* Blocks	0.14	0–0.29
Residual	0.15	0.11–0.24
Fertilizer *B*: 0–20–20, Powdered		
Potash		
Blocks[c]	—	—
Days *w.* Blocks	0	0–1.73
Bags *w.* Blocks	1.19	0.51–2.84
Samples *w.* Bags	0	0–0.32
Bags × Days *w.* Blocks	0	0–0.35
Residual	0.28	0.18–0.56
Fertilizer *C*: 5–10–10, Powdered		
Nitrogen		
Blocks[d]	0.04	0–1.70
Days *w.* Blocks	0.04	0.01–0.27
Bags *w.* Blocks	0.04	0–0.14
Samples *w.* Bags	0.04	0.01–0.08
Bags × Days *w.* Blocks	0.03	0–0.07
Residual	0.04	0.03–0.07
Total Phosphorus (P_2O_5)		
Blocks[c]	—	—
Days *w.* Blocks	0.05	0–2.32
Bags *w.* Blocks	0.13	0–0.37
Samples *w.* Bags	0.04	0–0.17
Bags × Days *w.* Blocks	0.09	0–0.27
Residual	0.10	0.07–0.20
Available Phosphorus (P_2O_5)		
Blocks[c]	—	—
Days *w.* Blocks	0.02	0–1.70
Bags *w.* Blocks	0.09	0–0.31
Samples *w.* Bags	0.05	0–0.17
Bags × Days *w.* Blocks	0.12	0–0.31
Residual	0.09	0.06–0.18
Potash		
Blocks[c]	—	—
Days *w.* Blocks	0	0–1.92
Bags *w.* Blocks	0.12	0–0.30
Samples *w.* Bags	0.04	0–0.17
Bags × Days *w.* Blocks	0	0–0.11
Residual	0.12	0.08–0.24

[a] Based only on part of the data, since the remainder was not homogeneous. *See* p. 882.
[b] Laboratories 2 and 3 only.
[c] Based on Laboratory 3 only.
[d] Based only on Laboratories 1 and 3.

Table VI-2. Estimates of net standard deviations based on riffles

Source of Variation	Estimated Net Standard Deviation (%)	Estimated 0.95 Confidence Limits (%)
Fertilizer *A:* 10–10–10, Granulated		
Nitrogen		
Blocks	0.33	0.15–2.06
Bags *w.* Blocks	0.09	0–0.19
Samples *w.* Bags[a]	0.15	0.12–0.21
Total Phosphorus (P_2O_5)		
Blocks	0	0–0.65
Bags *w.* Blocks	0.45	0.29–0.69
Samples *w.* Bags[a]	0.06	0.05–0.08
Available Phosphorus (P_2O_5)		
Blocks	0	0–0.54
Bags *w.* Blocks	0.41	0.26–0.63
Samples *w.* Bags[a]	0.06	0.05–0.09
Potash		
Blocks	0	0–0.09
Bags *w.* Blocks	0.46	0.29–0.72
Samples *w.* Bags[a]	0.18	0.14–0.25
Fertilizer *B:* 0–20–20, Powdered		
Total Phosphorus (P_2O_5)		
Blocks	0.08	0–1.61
Bags *w.* Blocks	0.64	0.36–1.04
Samples *w.* Bags[a]	0.41	0.32–0.57
Available Phosphorus (P_2O_5)		
Blocks	0	0–1.13
Bags *w.* Blocks	0.63	0.36–1.03
Samples *w.* Bags[a]	0.39	0.31–0.55
Potash		
Blocks	0.26	0–2.55
Bags *w.* Blocks	0.83	0.48–1.33
Samples *w.* Bags[a]	0.48	0.38–0.67
Fertilizer *C:* 5–10–10, Powdered		
Nitrogen		
Blocks	0.10	0–0.76
Bags *w.* Blocks	0.18	0.11–0.29
Samples *w.* Bags[a]	0.10	0.08–0.13
Total Phosphorus (P_2O_5)		
Blocks	0	0–0.51
Bags *w.* Blocks	0.19	0.08–0.34
Samples *w.* Bags[a]	0.18	0.14–0.26
Available Phosphorus (P_2O_5)		
Blocks	0.09	0–0.55
Bags *w.* Blocks	0.18	0.07–0.26
Samples *w.* Bags[a]	0.19	0.18–0.33
Potash		
Blocks	0	0–0.53
Bags *w.* Blocks	0.19	0.09–0.32
Samples *w.* Bags[a]	0.16	0.12–0.22
Fertilizer *D:* 10–10–10, Granulated		
Nitrogen		
Blocks	0.26	0.16–1.67
Bags *w.* Blocks	0.13	0.05–0.23
Samples *w.* Bags[a]	0.13	0.10–0.19
Total Phosphorus (P_2O_5)		
Blocks	0.20	0–1.69
Bags *w.* Blocks	0.51	0.33–0.80
Samples *w.* Bags[a]	0.16	0.12–0.22
Available Phosphorus (P_2O_5)		
Blocks	0.29	0–2.14
Bags *w.* Blocks	0.48	0.31–0.76
Samples *w.* Bags[a]	0.15	0.12–0.21
Potash		
Blocks	0.17	0–1.90
Bags *w.* Blocks	0.54	0.03–1.00
Samples *w.* Bags[a]	0.21	0.16–0.29

[a] Actually a combination of within-bag variation and within-day laboratory test error.

unsymmetrical and when there is only one degree of freedom, the confidence limits are practically worthless. These are reported simply to complete the record.

On the whole, the results agree reasonably well but there are several striking differences. The test error for potash (i.e., variation between duplicates within the same day) seems to run much higher when derived as a residual in a more complex analysis than when measured directly as the difference between duplicates on the same day. This will be seen to be true for both Fertilizers A and B, which are the only fertilizers for which this comparison is available.[13] An explanation is not readily available.

[13] For Fertilizer B this result was previously noted in Chapter IV, Section 3.

Table VI-3. Comparison of estimates of various net standard deviations

FERTILIZER A: 10–10–10 GRANULATED

Between Laboratories or Blocks

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9[a]	df	Table A-11	df	Table VI-2	df
N	0.06–1.97	2	0–10.43	1	0.15–2.06	2
TP	0–1.11	2	0– 0.39	2	0–0.65	2
AP	0.06–1.41	2	0– 0.61	2	0–0.54	2
K	0–0.14	2	0– 0.97	2	0–0.09	2

Between Days Within the Same Laboratory

Chemical Element	Estimated 0.95 Confidence Limits From: Table IV-3	df	Table A-11	df
N	0.05–0.21	6	0–0.35	2
TP	—	—	0.03–0.28	3
AP	0.01–0.13	6	0.02–0.25	3
K	0.02–0.12	6	0–0.10	3

Between Duplicates Within the Same Day

Chemical Element	Estimated 0.95 Confidence Limits From: Table IV-5	df	Table A-11	df
N	0.08–0.14	21	0.08–0.19	10
TP	—	—	0.07–0.13	21
AP	0.06–0.11	21	0.06–0.11	18
K	0.05–0.09	21	0.09–0.17	21

Between Samples Within the Same Bag

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9[b]	df	Table A-11[d]	df	Table VI-2[e]	df
N	0.13–0.36	9	0–0.14	10	0.12–0.21	24
TP	0.06–0.16	9	0–0.10	21	0.05–0.08	24
AP	0.07–0.19	9	0–0.11	18	0.05–0.09	24
K	0.04–0.11	9	0–0.16	21	0.14–0.25	24

Between Bags Within the Same Manufacturer's Lot

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9	df	Table A-11	df	Table VI-2	df
N	0–1.65	2	0.13–0.61	10	0–0.19	21
TP	0.10–2.17	2	0.32–0.82	21	0.29–0.69	21
AP	0.11–2.03	2	0.30–0.85	15	0.26–0.63	21
K	0–0.99	2	0.35–0.92	21	0.29–0.72	21

Table VI-3 (*Continued*)

FERTILIZER B: 0–20–20 POWDERED

Between Laboratories or Blocks

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9	df	Table A-11	df	Table VI-2	df
TP	0–1.16	2	0–1.51	1	0–1.61	2
AP	0–1.85	2	0–7.36	1	0–1.13	2
K	0–0.36	2	—	—	0–2.55	2

Between Days Within the Same Laboratory

Chemical Element	Estimated 0.95 Confidence Limits From: Table IV-3	df	Table A-11	df
TP	—	—	0–0.01	2
AP	0.15–0.42	6	0–0.05	2
K	0–0.19	6	0–1.73	1

Between Samples Within the Same Bag

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9[b]	df	Table A-11[d]	df	Table VI-2[e]	df
TP	0.12–0.32	9	0.05–0.31	14	0.32–0.57	24
AP	0.12–0.32	9	0–0.29	14	0.31–0.55	24
K	0.16–0.42	9	0–0.32	7	0.38–0.67	24

Between Duplicates Within the Same Day

Chemical Element	Estimated 0.95 Confidence Limits From: Table IV-5	df	Table A-11	df
TP	—	—	0.10–0.21	14
AP	0.09–0.16	21	0.11–0.24	14
K	0.10–0.18	21	0.18–0.56	7

Between Bags Within the Same Manufacturer's Lot

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9	df	Table A-11	df	Table VI-2	df
TP	0.13–2.51	2	0.50–1.65	12	0.36–1.04	21
AP	0.10–2.67	2	0.47–1.54	12	0.36–1.03	21
K	0.37–6.09	2	0.51–2.84	6	0.48–1.33	21

(Continued)

Table VI-3 (*Continued*)

FERTILIZER C: 5–10–10 POWDERED

Between Laboratories or Blocks

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9	df	Table A-11	df	Table VI-2	df
N	0.07–0.75	2	0–1.70	1	0–0.76	2
TP	0.04–0.99	2	—	—	0–0.51	2
AP	0–1.23	2	—	—	0–0.55	2
K	0–0.88	2	—	—	0–0.53	2

Between Days Within the Same Laboratory

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-11	df
N	0.01–0.27	2
TP	0–2.32	1
AP	0–1.70	1
K	0–1.92	1

Between Duplicates Within the Same Day

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-11	df
N	0.03–0.07	14
TP	0.07–0.20	7
AP	0.06–0.18	7
K	0.08–0.24	7

Between Samples Within the Same Bag

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9[b]	df	Table A-11[d]	df	Table VI-2[c]	df
N	0.03–0.09	9	0.01–0.08	14	0.08–0.13	24
TP	0.06–0.15	9	0–0.17	7	0.14–0.26	24
AP	0.08–0.20	9	0–0.17	7	0.18–0.33	24
K	0.10–0.26	9	0–0.17	7	0.12–0.22	24

Between Bags Within the Same Manufacturer's Lot

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9	df	Table A-11	df	Table VI-2	df
N	0.19–3.20	2	0–0.14	12	0.11–0.29	21
TP	0.13–2.29	2	0–0.37	6	0.08–0.34	21
AP	0.07–1.91	2	0–0.31	6	0.07–0.26	21
K	0–2.66	2	0–0.30	6	0.09–0.32	21

Table VI-3 (*Continued*)

FERTILIZER D: 8–16–16 GRANULATED

Between Laboratories or Blocks

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9	df	Table VI-2	df
N	0.09–1.81	2	0.16–1.67	2
TP	0–0.52	2	0–1.69	2
AP	0–0.73	2	0–2.14	2
K	0–0.79	2	0–1.90	2

Between Samples Within the Same Bag

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9[b]	df	Table VI-2[c]	df
N	0.06–0.17	9	0.10–0.19	24
TP	0.07–0.18	9	0.12–0.22	24
AP	0.07–0.19	9	0.12–0.21	24
K	0.07–0.20	9	0.16–0.29	24

Between Bags Within the Same Manufacturer's Lot

Chemical Element	Estimated 0.95 Confidence Limits From: Table A-9	df	Table VI-2[b]	df
N	0–0.83	2	0.05–0.23	21
TP	0.21–3.72	2	0.33–0.80	21
AP	0.18–3.61	2	0.31–0.76	21
K	0.21–4.10	2	0.03–1.00	21

[a] Laboratories are here treated as a random variable.
[b] Samples were obtained by riffling a composite made up of instrument samples from the same bag. The standard deviation is not "net" in this case, but includes laboratory test error and some day-to-day variation.
[c] Samples are two riffles of the same bag. Again, the standard deviation is not net, but includes laboratory test error. For computation, *see* Note 18 of the Appendix.
[d] Samples are two cores by same instrument from same bag, but taken from different corners.

Another striking difference is that between the confidence limits based on Table IV-3 and those based[14] on Table A 11 (*See* Appendix) for the day-to-day variation within the same laboratory for available phosphorus of Fertilizer B. The smallness in the day-to-day variations for available phosphorus of Fertilizer B as found from analysis of the results of Steps 13, 28, and 43 (on which Table A 11 is based) was noted previously.[15] It would seem now that they are definitely out of line with other results and are probably due to some exceptional set of conditions that existed at the time.

With respect to the within-bag sampling variation as measured by riffles, it will be

[14] Table A 11 is the basic table from which Table VI-1 was derived. For many parts of Table VI-3 reference can as easily be made to Table VI-1.

[15] *See* Chapter IV, Section 2, "Day-to-Day Variations as Estimated from the Supplementary Experiment."

Table VI-4. Build-up of tolerances for chemical components[a]

(Based on selection of 20 bags at random from a large lot, sampling with an unbiased sampling tube, compositing the 20 cores, reduction by riffling, and taking the mean of 2 chemical determinations run on separate days by laboratory selected at random)

	Fertilizer		
	A: 10–10–10, Granulated	B: 0–20–20, Powdered	C: 5–10–10, Powdered
1. Net Variance of Mean of 20 Cores			
N	0.0042	—	0.0002
TP	0.0130	0.0425	0.0010
AP	0.0113	0.0371	0.0006
K	0.0160	0.0704	0.0008
2. Net Variance of Riffling			
N	0.0021	—	0.0047
TP	0	0.0837	0.0199
AP	0	0.0939	0.0182
K	0.0220	0.0163	0.0025
3. Net Variance of Mean of Two Chemical Determinations on Separate Days by Same Laboratory			
N	0.0117	—	0.0017
TP	0.0072	0.0402	0.0065
AP	0.0048	0.0380	0.0042
K	0.0042	0.0114	0.0068
4. Net Interlaboratory Variance			
N	0.0893	—	0.0130
TP	0.0242	0.0272	0.0231
AP	0.0472	0.0724	0.0297
K	0	0	0
5. Total Variance			
N	0.1073	—	0.0196
TP	0.0444	0.1936	0.0505
AP	0.0633	0.2414	0.0527
K	0.0422	0.0981	0.0101
6. Total Standard Deviation			
N	0.33	—	0.14
TP	0.21	0.44	0.22
AP	0.25	0.49	0.23
K	0.21	0.31	0.10
7. One-Way 1-out-of-100 Tolerance			
N	0.77	—	0.33
TP	0.49	1.02	0.51
AP	0.58	1.14	0.53
K	0.49	0.72	0.23

Table VI-4 (*Continued*)

	Fertilizer		
	A: 10–10–10, Granulated	B: 0–20–20, Powdered	C: 5–10–10, Powdered
8. The Miles-Quackenbush One-Way 1-out-of-100 Tolerance[b]			
N	0.24	—	0.17
TP	0.40	0.44	0.40
K	0.40	0.57	0.40

[a] For method of computation, *see* Note 17 of Appendix. The tolerances are based on unbiased estimates of the various components of variance, but all are subject to considerable sampling errors. *See* Note 20 for discussion of the confidence limits of the various tolerances.
[b] *This Journal*, **38**, 120 (1955).

noted that the confidence limits bracket a distinctly higher range for potash than do the confidence limits for instrument samples. This suggests that the variability of riffles may be higher than that for instrument samples in the case of potash. This same phenomenon, however, was noted previously in Section 5 of Chapter III and was not found to be statistically significant.

2. Tolerances

It may be of some interest to use the components of variance presented in the previous section to compute allowances or tolerances for manufacturing variation and sampling and test error. This is done in Table VI-4 for each of the four chemical elements of Fertilizers A, B, and C. The results are based on compositing instrument samples from 20 bags chosen at random from a large lot (of 200 bags or more), taking a single riffle from this composite, choosing a laboratory at random and taking the mean of two laboratory determinations.

It is to be noted that the tolerances of Table VI-4 are merely suggestions as to the general order of magnitude of such tolerances. The computation of the tolerances of this table makes use of estimates of systematic laboratory variations that are based on only three laboratories and confidence limits for the "true" tolerances are consequently very large.[16] It is also to be noted that the tolerances of Table VI-4 run higher than those of Miles and Quackenbush[17] because of the inclusion of a component of variance for systematic laboratory error. This is particularly true for nitrogen.

Tolerances such as those offered in Table VI-4 may be of interest to large manufacturers who ship their fertilizer into many states. For in these cases they would probably not know what particular lots of fertilizer would be tested by what laboratories and hence may wish to make a general allowance for the systematic variation of control laboratories. The tolerances may also be of some interest to control laboratories that do not know their own systematic variations and therefore elect to make a general allowance for such variation. Usually, however, it is better both for the manufacturer and a control laboratory if that laboratory determines its own systematic variation as precisely as possible and then makes specific allowance for this systematic effect in its control procedure.

[16] *See* Note 19 of the Appendix.
[17] *This Journal* **38**, 108 (1955).

APPENDIX
of
STATISTICAL CALCULATIONS AND TECHNICAL MATERIAL

Notes

Appendix Tables

Appendix Tables (*Continued*)

Note 1. Instructions For Drawing the Sample of Fertilizer*

General Plan

The procedure will be the same for all 4 fertilizers, except that the random numbers will be different.

A stroke is a mark on the order-sheet that denotes 1 hand-truck. A hand-truck usually carries 6 bags, although a truck will occasionally be short, and one is occasionally long, especially at the end of an order.

Stroke | Group 𝍸

The primary unit for counting will be the group of 5 strokes. Number the groups of the 1st lot consecutively 1, 2, 3, etc., until the lot is exhausted. The 1st group of the next lot takes the next serial number. The random numbers in the table supplied herewith will select some of the groups. These will be the sample-groups.

A random number between 1 and 5 in the same table will select one hand-truck within a sample-group. The bags on a full hand-truck will have numbers 1, 2, 3, 4, 5, 6 bottom to top.

Draw off and set aside for the sample bag No. 6 from every hand-truck that the random numbers select.

The Sampling Table

The sampling table contains pairs of numbers, under the columns Group and Truck. The first number 0006 selects the 6th group. The number alongside it under Truck is 2, which selects the 2d hand-truck, or 2d stroke. Bag No. 6 thereon will be a sample-bag.

The random numbers will in some cases of incomplete groups and incomplete trucks lead to no sample (*see* paragraphs under

Sampling table for Fertilizer A (10–10–10 granulated)

Group	Truck	Group	Truck
0006	2	0772	5
0081	4	0799	3
0135	3	0892	1
0146	5	1003	5
0187	4	1036	5
0217	4	1104	3
0218	4	1134	3
0238	1	1222	1
0323	1	1383	1
0384	2	1447	4
0426	4	1488	5
0445	1	1616	4
0446	4	1634	3
0447	4	1748	5
0448	1	1814	1
0500	2	1958	2
0501	5	1977	4
0530	3	1987	3
0532	3		
0536	2		
0563	3		
0579	4		
0595	3		
0646	5		
0703	5		

* Prepared by W. Edwards Deming.

Exceptions and Blanks). In other words, a pair of random numbers will yield either a bag or a blank. Make no substitution for a blank; to do so will impair the sampling procedure. Merely mark the sampling table *BL* wherever it leads to a blank.

It is necessary to account for every pair of random numbers. The total number of bags drawn, plus the number of blanks, will equal the number of pairs of random numbers.

The tables supplied herewith will produce about 32 bags of each fertilizer, provided that the number of tons in the lot is about equal to the figure shown.

Exceptions and Blanks

Fill up with blanks to 5 any incomplete group. If a group contains only 3 trucks, then 4 and 5 are blanks; and if the random number for the truck in this group is 4 or 5, there will be no sample.

If a truck has fewer than 6 bags, use the auxiliary table to decide whether to draw a bag.

There will occasionally be a truck with 7 bags. A truck with 7 bags is denoted, not by a stroke, but by the figure 7. When such a truck comes into the sample, take the 6th bag, and use the auxiliary table to decide whether the 7th bag will also go into the sample; it may or may not. (Treat Bag No. 7 as No. 1 in a new series, of which Bags 2, 3, 4, 5, 6 are blanks.)

The Auxiliary Table

Use this table whenever a truck-load is incomplete (less than 6 bags). Number the bags 1, 2, 3, as far as they go; the other numbers to 6 are blank. If there were, for example, 2 bags on the truck, numbers 3, 4, 5, 6 would be blank. Random number 1 draws the bag off the bottom. Random number 2 draws Bag No. 2; etc. Random number 3, 4, 5, or 6 would draw a blank; no sample. Random number 6 will always draw a blank, as otherwise the truck would not be incomplete.

Make no substitution for a blank.

Mark each number on the auxiliary table as you use it, and use the next number the next time. When you have exhausted the table, begin at the front. Then use it over again.

Auxiliary Table

5	1	1	3	5	5
6	4	6	2	1	3
2	5	2	5	3	6
4	2	4	1	2	2
3	6	3	6	4	4
1	3	5	4	6	1

Note 2. Random Cycling of the Men and of the Instruments*

Notation

Laboratories: 1—Virginia
2—New Jersey
3—South Carolina

The numbers for the laboratories were drawn after the experiments were performed. The randomization of the instruments and of the men was made on the spot:

Instruments: *x*—single tube
y—double tube
z—Archer tube

Men:
1—Mr. F. L. Groendyke (New Jersey)
2—Mr. F. M. Herndon (So. Carolina)
3—Mr. E. H. Isbel (Virginia)
4—Mr. J. R. Archer (International Minerals & Chemical Corp.)

One will note from the detailed description of the experiment that the 4 men each draw 3 cores with 3 instruments, to perform Steps 1-12, 16-27, 31-42. The order (*1st, 2nd, 3rd, 4th*) for Dr. Archer's appearance was first of all alotted by random numbers. His instruments were then alotted at random (*see table*).

The other 3 men thereupon took the other 3 places at random. This randomization was carried out so that the 3 men and the 3 instruments that they used formed a Latin square for each laboratory. The description of the experiment in Note 3 shows the actual order of the men and of the instruments.

* Prepared by W. Edwards Deming.

The first 6 thrusts of the instruments in Steps 1-12, 16-27, and 31-42 for each fertilizer were from the corner of the bag at the man's left. The second 6 thrusts were from his right. The 2 thrusts into each bag in Steps 13, 28, and 43 alternated likewise in direction, the first one being from the man's left, the second from his right.

Order for Mr. Archer

(The numerals denote his order of appearance. The letters denote in order the instruments that he used.)

Laboratory	Fertilizer			
	A	B	C	D
1	2	3	1	4
	x	*y*	*z*	*y*
	y	*z*	*x*	*z*
	z	*x*	*y*	*x*
2	1	4	2	3
	z	*y*	*z*	*z*
	y	*x*	*x*	*y*
	x	*z*	*y*	*x*
3	4	2	3	1
	x	*x*	*y*	*y*
	z	*y*	*x*	*x*
	y	*z*	*z*	*z*

Note 3. Directions for Conduct of the Experiment *

Steps 1-15 are diagrammed in the accompanying flow-sheets.

Steps 16-30.—Bags 9-16, Laboratory 2.

Steps 31-45.—Bags 17-24, Laboratory 3.

Steps 16-27.—For Bag No. 9, same procedure as for Bag No. 1, Steps 1-12.

Steps 31-42.—Bag No. 17. Similar procedure as for Bag No. 1, Steps 1-12.

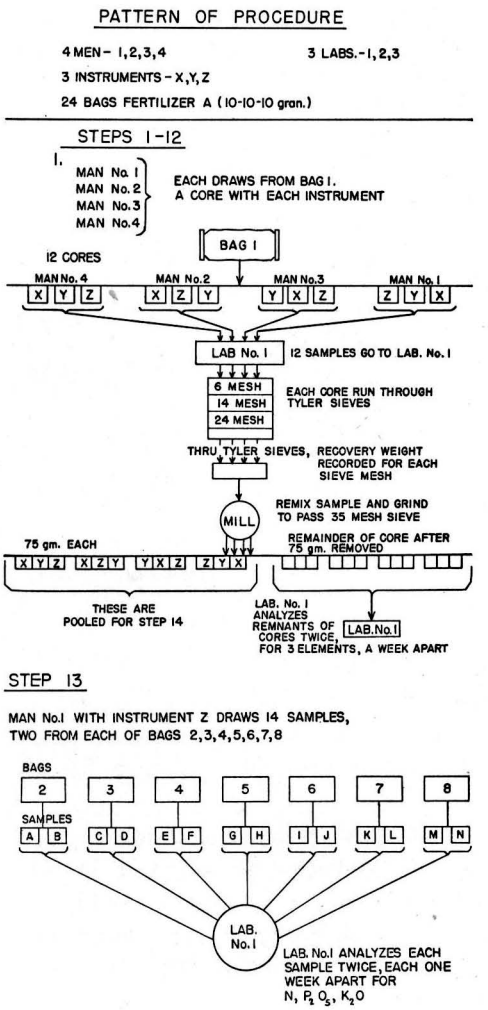
Step 28.—Similar procedure to Step 13.

Man No. 4 draws 14 samples with instrument *z*. 2 samples each from Bags 10, 11, 12, 13, 14, 15, 16. Each of these 14 samples goes to Laboratory No. 2 for tests a week apart on N, P_2O_5, K_2O.

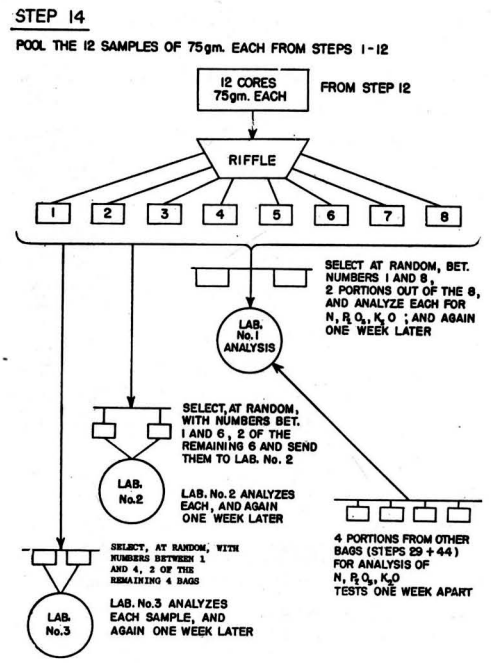
Step 43.—Similar procedure to Step 13.

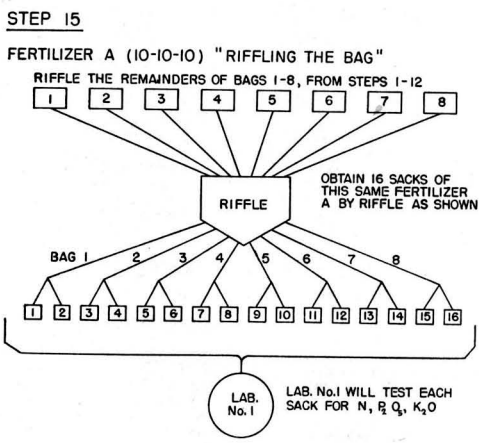
Man No. 1 draws 14 samples with instrument *x*. 2 samples each from Bags 18 to 24, inclusive. These 14 samples go to Laboratory No. 3 for tests one week apart on all three elements, N, P_2O_5, K_2O.

Step 30.—Similar procedure to Step 15 applied to Bags 9 to 16 inclusive, except that Laboratory No. 2 replaces Laboratory No. 1.



* Summarized by Vincent Sauchelli from original directions by W. Edwards Deming.





Step 45.—Similar procedure to Step 15, applied to Bags 17 to 24 inclusive, except that Laboratory No. 3 replaces Laboratory No. 1.

Step 29.—Laboratory No. 2 follows Step 29, similar to procedure Step 14, Laboratory No. 1.

Step 44.—Laboratory No. 3 follows Step 44, similar to procedure Step 14, Laboratory 1.

Almost identical procedures were followed for Fertilizers B, C, D, except that tests one week apart were not carried out for all chemical elements. *See* text, page 853.

Note 4. General Directions to the Laboratories

A laboratory will test each sample once or in duplicate, whichever is the routine practice. For tests in duplicate, the laboratory will report both results, not merely the average. If the laboratory repeats a test for any reason, it will report all the results, in order, with the reason for repeating the test.

Note 5. Table of Loads for the Laboratories*

Laboratory 1

Fertilizer	N	P	K	Sieve
A	68	68	68	12
B	0[a]	48	48	0
C	68	48	48	0
D	48	48	48	12
All 4	184	212	212	24

Laboratory 2

Fertilizer	N	P	K	Sieve
A	68	68	68	12
B	0[a]	68	48	0
C	48	48	48	0
D	48	48	48	12
All 4	164	232	212	24

Laboratory 3

Fertilizer	N	P	K	Sieve
A	68	68	68	12
B	0[a]	48	68	0
C	48	48	48	0
D	48	48	48	12
All 4	164	212	232	24

[a] Fertilizer B is 0–20–20.
* Computed by W. Edwards Deming.

Note 6. Editing the Data

The directions to the laboratories allowed for possible reporting of several results per test (*See* Note 4). Altogether there were 39 instances in which more than one result per test was reported and it was necessary for the statistician to make some decision on these extra data.

When more than one result was reported, the first was used in the initial analysis of the data. This initial analysis provided an original estimate of the net day-to-day variation in laboratory tests. It was recognized that this day-to-day variance was based in part on some of the data on which it would ultimately be used to pass judgment. The effect of the contamination, however, was believed to be small and the gain from rerunning the analysis without the suspected data (which would be greatly complicated because of lack of orthogonality) was not deemed to be worth the extra expense.

The supplementary experiment (*See* Chapter I, Section 2, "Intra-laboratory Variations") yielded information on the variance between duplicates run the same day. None of these supplementary data involved more than one determination per test and hence were presumed to be uncontaminated.

The original estimate of net day-to-day variance and the estimate of within-day variance were added together to yield an estimate of the variance of test results run on separate days. The square root of this combined variance was multiplied by a 0.05 studentized range factor for comparing two or three results, whichever was appropriate, the factor being based on an estimate of the appropriate degrees of freedom. If the range of a set of two (or three) results did not exceed this studentized range factor, the data of the set were assumed to be homogeneous, otherwise they were taken to be significantly different. Of the 39 sets of multiple test results, 33 were judged to be homogeneous and 6 not.

If a set of multiple test results was judged to be homogeneous, the first result was used in the revised statistical analysis. If a set of two were judged to be significantly different, the result which was closer to the nominal value was the one used in the revised analysis, the other being viewed as an "incorrect" determination. If, in a set of three, one was judged to be significantly different, the result used was the first of the two that were closest.

Note 7. Effect of Order of Using Instruments

At the time the experiment was designed, it was thought that the order in which the three instruments were used in the bag might have some effect on the results. It was believed, however, that this effect would be small and the order of use of the instruments was therefore randomized so that any such effects could be merged with the experimental and sampling errors in the statistical analysis. Nevertheless, part of the experiment was so arranged that three of the men, the three instruments, and their three orders of use would form a Latin square so that the actual effects of order could be determined. The statistical analyses of the various Latin squares is given in Tables A 1 and A 2. It will be noted that no significant order effects were found in any of the four fertilizers.

Note 8. Analysis of Variance of Sieve Weights and Chemical Determinations with Order of Sampling Randomized

The analysis of variance of sieve weights with order of sampling randomized takes the form of a 3×4 factorial with 3 replications, for each of the three instruments was used by each of the four men and the whole was replicated three times. The same experiment was run for each of the four fertilizers, but only the two granulated fertilizers were subjected to sieve analysis.

In the analysis of variance the instruments and men were both viewed as being "fixed factors." Accordingly, the F-ratios were

computed for all main effects and interactions by comparing their mean squares directly with the error mean square. These ratios are shown in Table A 3, together with the mean squares from which they were computed.

From Table A 3 it will be noted that in the case of Fertilizer A there are significant differences between the instruments for both the 6 mesh and 24 mesh and in the case of Fertilizer D for the 6 mesh.

The analysis of variance of chemical determinations takes the same general form as that of sieve weights, since the data came from the same experiment. Here, however, the analysis covers all four types of fertilizer. The various mean squares and F-ratios are presented in Table A 4.

It will be noted from Table A 4 that the only significant instrument effect is that for nitrogen in the 8-16-16 fertilizer. The significant differences in sieve weight found in Fertilizer A do not show up in the chemical analysis.

Note 9. Analysis of Variance of Instrument Sampling *vs.* Riffling

The analysis of variance here is that of a "mixed," "nested" model. A block of tests consists of those resulting from the use of a different sampling instrument, a different man, and a different laboratory. Within each block, the mean (actually total) of two instrument samples from each of seven bags of fertilizer, selected at random from the lot, is compared with the mean (actually total) of two riffles from the same bag. Each block has a different set of bags. The bags are thus a "random factor" nested in blocks and "cross-classified" with type of sampling.

The basic data are given in Table A 5 and the mean squares and F-ratios are presented in Table A 6. The F-ratio for the Block × Type-of-Sampling interaction is the ratio of the Block × Type mean square to the residual mean square. The F-ratio for Blocks is the ratio of the mean square for Blocks to the mean square for Bags-within-Blocks, and the F-ratio for Type-of-Sampling is the ratio of the mean square for Type to the residual mean square. For the purposes of this part of the experiment the "blocks" were viewed as fixed factors, the mean effects of which were "balanced out" by the experimental design.

It will be noted that there are significant mean differences between types of sampling (i.e., significant relative biases between instrument samples and riffles) for nitrogen, available phosphorus, and potash of Fertilizer C, and also for potash of Fertilizer B and potash of Fertilizer D. In the case of Fertilizers C and D the F-ratios Type/Residual for all the other chemical elements are significant at the 0.10 level (i.e., they have less than a 1 out of 10 probability of occurring by chance), but not at the 0.05 level. If these particular results are of special interest, more conclusive evidence might be obtained by further experimentation.

Further evidence of significant effects of type of sampling is yielded by the high F-ratios for Block × Type interactions. These are for phosphorus in Fertilizers B and D as well as for potash of Fertilizers C and D. The results here indicate that the different types of sampling showed up differently in the different blocks, which may or may not have been due to the use of different sampling instruments, a result that also might be the subject of further investigation.

It will be noted that in the case of the first week's tests on Fertilizer A, there are neither significant main effects nor significant interactions for any chemical elements. Table A 7 shows, however, that in the tests run a week later on Fertilizer A, there are significant interactions for phosphorus between Type of Sampling and Blocks; this suggests that Type of Sampling does have some effect in sampling of Fertilizer A, but that this differs from block to block, the same point made above.

Note 10. Analysis of Variance of Absolute Within-Bag Sample-to-Sample Differences: Instrument Sampling *vs.* Riffling

The analysis of variance model for Table A 8 is the same as for Tables A 6 and A 7. The data, however, are now absolute differences instead of means (or totals). The means and ranges of these absolute differences are presented in Tables III-8 to III-11. The analysis of variance is carried out in the same manner as described in Note 9.

Table A 8 shows no significant mean difference between types of sampling (i.e., no significant mean difference in the precision of instrument samples and the precision of riffles) for any chemical element in any of the four fertilizers. In the case of phosphorus of Fertilizer B, there is a significant Type × Block interaction which suggests there may be a difference in the precision of the two types, but if there is, it is not the same for all blocks. A more plausible explanation is to assume variation in block effects that are possibly due to erratic variation in laboratory procedure.

It should be noted in conclusion that the analyses of variance presented in Table A 8 assume a homogeneous residual variance. That this may not be valid for the data in hand is strongly suggested by Figs. A 1—A 4, especially Fig. A 2. Note 11 studies this further with the conclusion that the residual variance is not homogeneous throughout the various parts of the experiment. Nothing is unearthed in Note 11, however, that would lead to a serious questioning of the conclusions drawn from Table A 8 based on the assumption of homogeneity of variances.

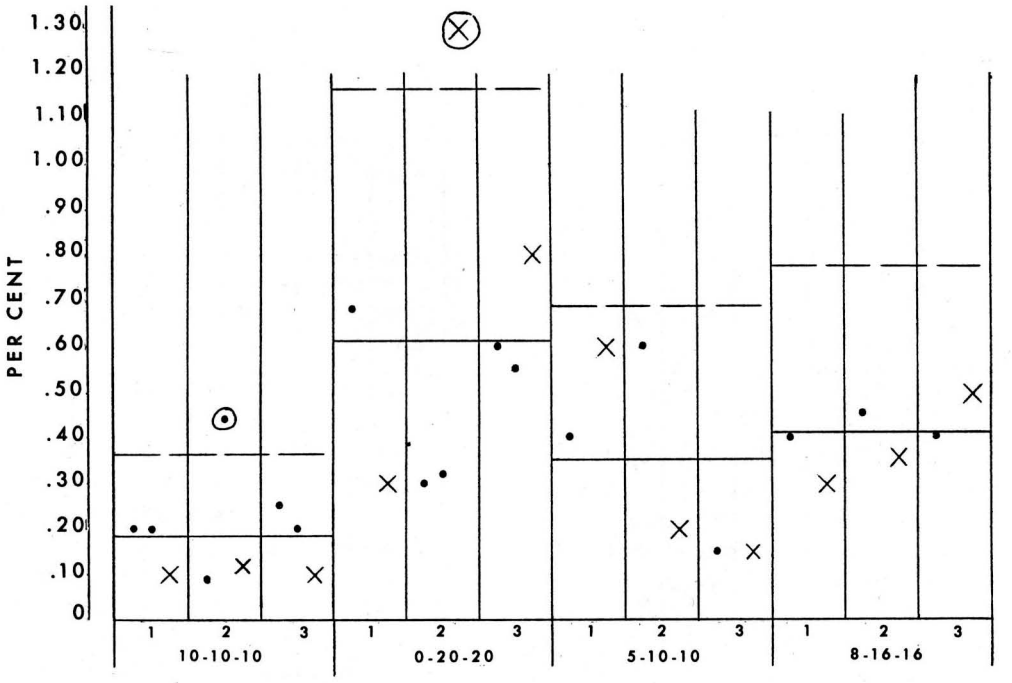


Fig. A-1—Ranges of absolute sample-to-sample differences: Total phosphorus.
Key: • = instrument samples; X = riffles.

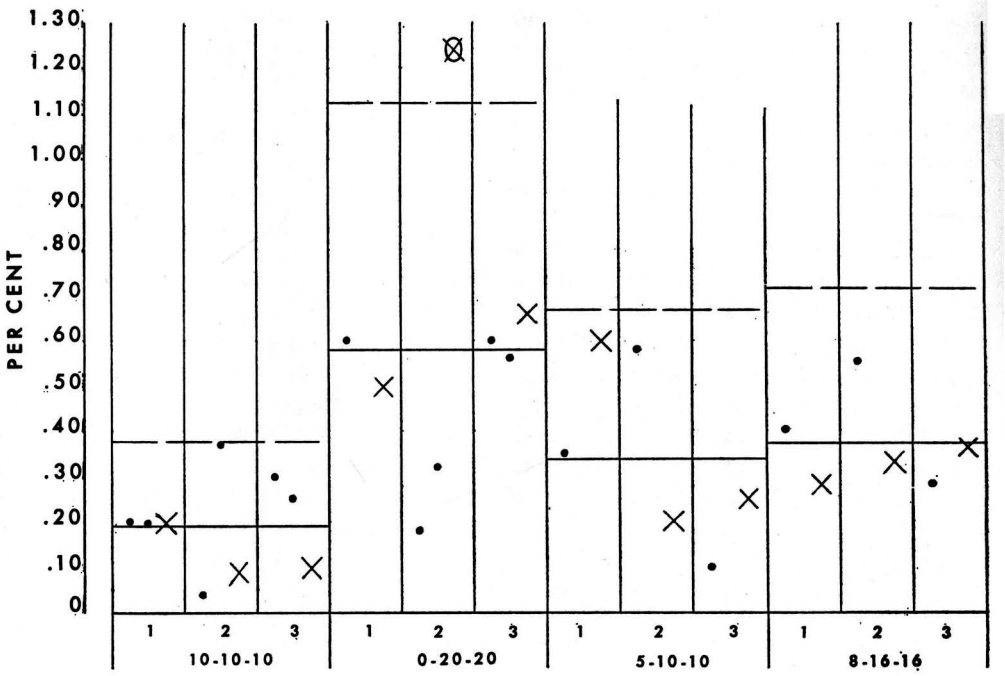


Fig. A-2—Ranges of absolute sample-to-sample differences: Available phosphorus.
Key: • = instrument samples; X = riffles.

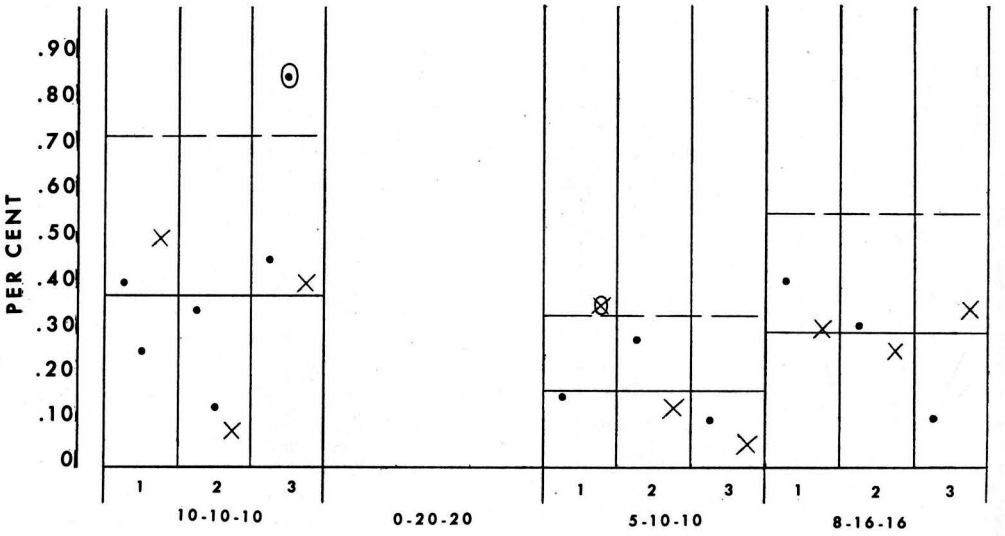


Fig. A-3—Ranges of absolute sample-to-sample differences: Nitrogen.
Key: • = instrument samples; X = riffles.

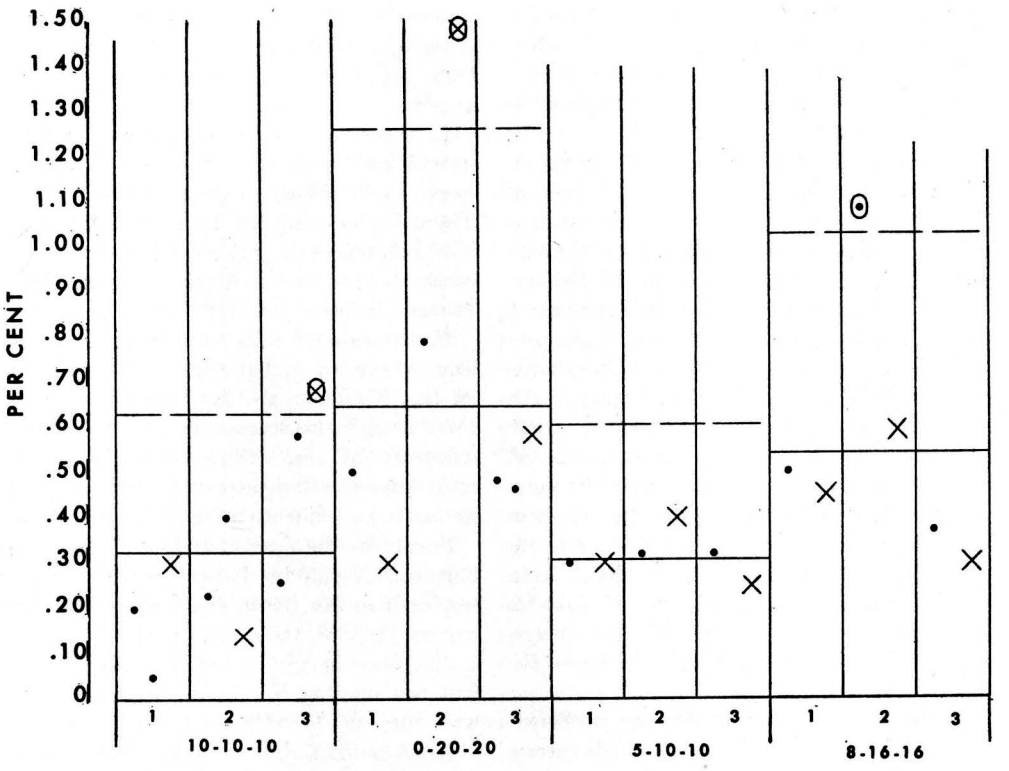


Fig. A-4—Ranges of absolute sample-to-sample differences: Potash.
Key: • = instrument samples; X = riffles.

Note 11. The Heterogeneity of the Variation of Within-Bag Sample Differences

A study of Figs. A 1 to A 4 suggests that the variation in differences between samples from the same bag might be greater than could reasonably be attributed to chance. In Fig. A 2, for example, the variation in the first week's analyses run by Laboratory 2 is exceptionally small, while the variation in the second week's analyses run on the same samples by the same laboratory is exceptionally high.

To study this variation in absolute sample differences, range "control charts" were drawn. These are simply plots of the ranges of the absolute sample differences given in Tables III-8 to III-11, together with lines showing the means and upper 3-sigma control limits. (Lower control limits were not presented. *See* Figs. A 1—A 4.) Since several points are outside the upper control limit, the conclusion is to be drawn that the variation in absolute sample differences is not "in control," as suggested above, or in other words, the variation is being affected by other than chance forces.

It was at first thought that the "lack of control" indicated by Figs. A 1—A 4 might be due to possible failure of the laboratories to run every analysis in a set of tests on the same day. An examination of the record, however, showed that each set of riffle tests was without exception run on a single day. For example, in the case of Fertilizer A the 14 nitrogen tests by Laboratory 1 on the riffles of bags No. 2 to No. 8 were all run on November 12, 1957; the 14 potash tests by Laboratory 3 on the riffles of bags No. 18 to No. 24 were all run on November 22, 1957; etc. Instrument samples were not so

homogeneous in this respect, however. Nevertheless, study of the results failed to yield any reason to believe that the heterogeneity was the cause of the out-of-control points of Figs. A 1—A 4.

A review of the homogeneity of test conditions yielded the information that lack of homogeneity in test conditions for instrument samples was confined to tests of available phosphorus in Fertilizer A by Laboratory 2 and tests of nitrogen in Fertilizer A by all laboratories.

In the case of tests on available phosphorus in Fertilizer A run by Laboratory 2, the determination of insoluble AP in the first sample of Bag No. 10 was made on 1/21/58 and that in the second sample from the same bag was run on 1/24/58. The difference was 0.03. In the case of Bags No. 11 to No. 16, tests were all run on the same day. These yielded four other differences of 0.03, 2 differences of 0.04 and one of 0.05. There is no evidence here that the difference for Bag No. 10 is out of line.

In the case of tests of nitrogen in Fertilizer A run the second week by Laboratory 1, the test on the first sample from Bag No. 4 was run on 11/19/57 and that on the second sample was run on 11/20/57. The difference was zero. Among the other six differences, there was one other difference of zero, four differences of 0.10 and one of 0.20. Again there is no evidence of heterogeneity.

In the case of tests on nitrogen in Fertilizer A run by Laboratory 2, the determinations (with dates) were as given below. There is no evidence here that the absolute differences are more or less alike because the tests were run on different dates. (*t*-tests indicate no significant differences.)

In the case of tests of nitrogen in Fertilizer A run by Laboratory 3, the first test on Bag No. 24 in the first week was run on 11/6/58 and the second on 11/7/58, with a difference of zero. Since there is another zero difference for tests run the same day, we have no evidence here of heterogeneity.

Finally in the case of tests of nitrogen in Fertilizer A run by Laboratory 3, the first test on Bag No. 20 in the second week was run on 11/6/58, the second on 11/7/58, with a difference in yield of 0.30 . Likewise, the first test on Bag No. 23 in the second week was run on 11/6/58 and the second on 11/7/58 with a difference in yield of 0.10. Other differences for the second week between sample tests run the same day were two 0.05's, another 0.10, one 0.15 and one 0.25. Again there is no evidence that abso-

Bag No.	Sample	First Week		Absolute Difference	Second Week		Absolute Difference
		Date	Result		Date	Result	
10	a	1/21/58	9.66		1/28/58	9.95	
	b	1/24/58	9.74	.08[a]	1/31/58	9.91	.04[a]
11	a	1/21/58	9.61		1/28/58	9.78	
	b	1/24/58	9.95	.34[a]	1/31/58	9.86	.08[a]
12	a	1/24/58	9.86		1/31/58	9.82	
	b	1/24/58	9.70	.16	1/31/58	9.82	0
13	a	1/24/58	9.74		1/31/58	9.86	
	b	1/24/58	9.95	.21	1/31/58	9.95	.09
14	a	1/24/58	9.83		1/31/58	9.70	
	b	1/21/58	9.83	0[a]	1/28/58	9.82	.12[a]
15	a	1/24/58	9.78		1/31/58	9.74	
	b	1/21/58	9.70	.08[a]	1/28/58	9.61	.13[a]
16	a	1/24/58	9.53		1/31/58	9.53	
	b	1/21/58	9.66	.13[a]	1/28/58	9.66	.13[a]

[a] Tests run on different dates.

lute differences were larger or smaller because the tests were run on different dates.

It would seem fair to conclude from the above that the heterogeneity revealed in Figs. A 1 to A 4 is not due to some of the tests in certain sets being run on different dates from the others. Since it is difficult on *a priori* grounds to believe that the heterogeneity could be caused by the use of different instruments or the use of the same instrument by a different man or some combination of the two, it would appear by deduction that it was due to unknown variations in laboratory testing conditions. It is possible that grinding fertilizer to pass a 35 mesh sieve does not make it homogeneous enough to eliminate sizeable sampling errors from the ground fertilizer and that shaking the bottle or digging into the bottle at different depths on different occasions might cause variations of the kind shown in Figs. A 1 to A 4.

Note 12. Analysis of Variance of Interlaboratory Comparisons

The mean squares and F-ratios for the analyses of variance of the interlaboratory comparisons are presented in Table A-9. The basic model is a "mixed" one; *viz.*,

$$Y = m + L_j + b_i + (Lb)_{ij} + e_{ijk} \begin{cases} j = 1,2,3 \\ i = 1,2,3 \\ k = 1,2 \end{cases}$$

where m is an over-all constant, L_j is the "fixed" systematic effect of laboratory j, b_i is the "random" variation of bag i, $(Lb)_{ij}$ is the interaction of L_j and b_i, and e_{ijk} is the sampling and test error associated with the test by laboratory j of the kth sample from bag i. The variable e_{ijk} is assumed to be normally distributed with zero mean and constant variance σ^2.

On the basis of the above model, the F-ratio for the laboratory main effect is computed by taking the ratio of the laboratory mean square to the interaction mean square; the F-ratio for the bag main effect is computed by taking the ratio of the bag mean square to the error mean square (since bags are assumed to be a random factor); and the F-ratio for the interaction effect is computed by taking the ratio of the interaction mean square to the error mean square.

The F-ratio for the bag main effect does not strictly follow the F-distribution[18], but the approximation is believed to be sufficiently valid for the purposes of the analysis.

It will be noted that in many cases either the laboratory main effects or laboratory-bag interactions are significant at the 0.05 level or better. The significant laboratory main effects suggest definite laboratory systematic effects, due possibly to methods or technicians. The significant laboratory-bag interactions suggest that the laboratories have systematic variations relative to each other but are not consistent in these systematic variations. Since each laboratory usually tested its two samples from a given bag on the same day, the significant interactions may simply be evidences of a day-to-day effect that might have shown up even if all the tests were run by the same laboratory. This day-to-day effect is studied in more detail in Section 2 of Chapter IV.

In conclusion it should be noted that the hypothesis of homogeneity of error variances between laboratories was tested in each case[19] and in only one instance was it rejected. This was in the analysis of total phosphorus of Fertilizer A. Since eleven such tests were carried out with only one rejection at the 0.05 level, the question may be raised as to whether even this one "significant" result might not be due to chance. The homogeneity of variances was in each case tested by using the ratio of the maximum mean square to the minimum mean square.

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[18] Scheffe, H.; "Alternative Models for the Analysis of Variance," *Annals of Mathematical Statistics*, Vol. XXVII (1956) pp. 251-71; and Duncan, A. J., *Quality Control and Industrial Statistics*, 2nd Ed., p. 559 ff.

[19] *A priori* there was some reason to believe that the error variances were not homogeneous since in a few cases tests on the two samples from a given bag were run on different days. A close examination of the data on which Table A 9 is based yielded very little evidence that *for these data* day-to-day influences were affecting the results *within* laboratories, although subsequent analyses do indicate the existence of such influences.

Note 13. Estimation of Net Standard Deviations of Day-to-Day Variation from Two Days a Week Apart

In Table IV-2, the estimate of day-to-day difference is the sum of the true day-to-day difference and the difference between two means of r_i test errors each. In mathematical terms we have:

Estimated Day-to-Day Difference——
$= d_1 - d_2 + \bar{e}_1 - \bar{e}_2$, where d_i is the variation attributable to the conditions of the ith day. If we assume the day-to-day variations, d_i, are randomly distributed around zero with variance of σ_d^2, then:

r_i (Estimated Day-to-Day Difference)^2 is an unbiased estimate of $2r_i\sigma_d^2 + 2\sigma_e^2$.
Hence, this minus an unbiased estimate of $2\sigma_e^2$ will give an unbiased estimate of $2r_i\sigma_d^2$ and the result divided by $2r_i$ will give an unbiased estimate of σ_d^2. Its square root will be an estimate of σ_d. This is the result given in the third column of Table IV-2. In each case the estimate of $2\sigma_e^2$ is taken as the pooled residual mean square for each chemical element. In the case of potash of Fertilizer A, only the residual mean squares for Laboratories 1 and 2 were pooled, since the residual mean square for Laboratory 3 was significantly different from those for Laboratories 1 and 2.

Note 14. Computation of Confidence Limits for the Standard Deviations of Day-to-Day Variation

Table IV-3 gives 0.95 confidence limits for the standard deviation of day-to-day variation as estimated from the supplementary experiment. The method of computation may be explained with reference to the per cent nitrogen in Fertilizer A.

The day-to-day mean square for the per cent nitrogen in Fertilizer A was computed to be 0.0365. This is an average of the day-to-day mean squares for all three laboratories and is based on 18 degrees of freedom since each laboratory replicated its tests for seven days. The residual mean square was computed to be 0.0100. This is the mean square of the variation between duplicates on the same day averaged over all seven days and over all three laboratories. The *net* variance for day-to-day variation is estimated at $(0.0365 - 0.0100)/2 = 0.0133$ and the net standard deviation is the square root of this or 0.115.

If we call the estimate of the net variance for the day-to-day variation s_1^2 and the residual mean square s_2^2, then 0.95 confidence limits for the ratio σ_1^2/σ_2^2 will be given by:

$$\frac{s_1^2}{2F_{0.025}(18,21)s_2^2} - \frac{1}{2} \leq \frac{\sigma_1^2}{\sigma_2^2} \leq \frac{s_1^2F_{0.025}(21,18)}{2s_2^2} - \frac{1}{2},$$

where σ_1^2 and σ_2^2 are the population day-to-day net variances for day-to-day variation and test error respectively. (*cf.* Duncan, A. J., *Quality Control and Industrial Statistics*, 2nd Ed., p. 624). Approximate confidence limits for σ_1^2 will be given by taking $\sigma_2^2 = s_2^2$ and multiplying each side of the equality by s_2^2. This yields:

$$\frac{s_1^2 - s_2^2F_{0.025}(18,21)}{2F_{0.025}(18,21)} \leq \sigma_1^2 \leq \frac{s_1^2F_{0.025}(21,18) - s_2^2}{2}$$

For the given values of s_1^2 and s_2^2, we have:

$$\frac{0.0365 - (0.0100)(2.47)}{2(2.47)} \leq \sigma_1^2 \leq \frac{(0.0365)(2.69) - 0.0100}{2}, \text{ or}$$

$$0.002388 \leq \sigma_1^2 \leq 0.04414,$$

and 0.95 confidence limits for the net standard deviation will be:

$$0.049 \leq \sigma_1 \leq 0.210$$

Confidence limits for other net standard deviations in Table IV-3 were computed in the same way.

Note 15. Determination of Confidence Limits for the Standard Deviations of Within-Day Variation

In Tables IV-4 and IV-5, 0.95 confidence limits for the various within-day standard deviations were obtained in the customary manner. The sum of the squared deviations from the sample mean was in each case divided by the 0.975 and 0.025 values of χ^2 for the degrees of freedom involved and the square root taken of the result. For a group of 2 or 3 laboratories, the sums of the squared deviations from the sample means were pooled and the grand sum divided by the sum of the degrees of freedom. In Table IV-4, the sums of the squared deviations were divided by two times the 0.975 and 0.025 χ^2 values, since we were interested in the standard deviations of the within-day variation, whereas the original day-to-day differences (a week apart) are differences between two such variations. When the degrees of freedom summed to more than 30, it was assumed that $(\sqrt{2\chi^2} - \sqrt{2n-1})$

was normally distributed with zero mean and unit variance where n equals the degrees of freedom. For the day-to-day data a week apart this led to the formula:

0.95 confidence limits for

$$\sigma = \frac{\sqrt{\Sigma \text{ Sq. Deviations}/(2n-1)}}{1 \pm 1.96/\sqrt{2n-1}}$$

(Note that under the radical sign, the 2's cancel out.)

Note 16. Tables of Bagging Variations

Detailed tables of bagging variations are presented in Table A-10. After these data were adjusted for differences in laboratory means, selected sets of the data were plotted in Fig. V-1 to V-4. The data in Table A-10 are means of two riffles from the same bag.

Note 17. The Analysis of Variance of the Data of Steps 13, 28, and 43 and Estimates of Components of Variance

The model that fits these data is the following:[20]

$X_{ijkl} = m + a_i + b_{ij} + c_{ijk} + d_{il} + (bd)_{ijl} + e_{ijkl}$, where:

m is an overall constant,
a_i is the effect of block i,
b_{ij} is the effect of the jth bag in block i,
c_{ijk} is the effect of the kth sample in bag j in block i,
d_{il} is the effect of the lth day in block i,
$(bd)_{ijl}$ is the interaction effect of the jth bag in day l in block i, and
e_{ijkl} is a random error.

All effects are viewed as being random.

For this model the expected values of the various mean squares are as follows where n_b is number of bags, n_c is number of samples per bag, and n_d is number of days:

Symbol Used	*Mean Square*	*Expected Value*
s_1^2	Bet. Blocks	$n_b n_c n_d \sigma_a^2 + n_c n_d \sigma_b^2 + n_d \sigma_c^2 + n_b n_c \sigma_d^2 + n_c \sigma_{bd}^2 + \sigma^2$
s_2	Bet. Days w. Blocks	$n_b n_c \sigma_d^2 + n_c \sigma_{bd}^2 + \sigma^2$
s_3^2	Bet. Bags w. Blocks	$n_c n_d \sigma_b^2 + n_d \sigma_c^2 + n_c \sigma_{bd}^2 + \sigma^2$
s_4^2	Bet. Samples w. Bags	$n_d \sigma_c^2 + \sigma^2$
s_5^2	Bags × Days w. Blocks	$n_c \sigma_{bd}^2 + \sigma^2$
s_6^2	Residual	σ^2

The components of variance are computed as follows:

Bags × Days w. Blocks	$(s_5^2 - s_6^2)/n_c$
Samples w. Bags	$(s_4^2 - s_6^2)/n_d$
Bags w. Blocks	$(s_3^2 - s_4^2 - s_5^2 + s_6^2)/n_c n_d$
Days w. Blocks	$(s_2^2 - s_5^2)/n_b n_c$
Blocks	$(s_1^2 - s_2^2 - s_3^2 + s_5^2)/n_b n_c n_d$

Since in the case of Fertilizer A not all bags in a given set were tested on the same day, part of the data that might have been used in the analysis of variance for available phosphorus and nitrogen had to be discarded. (See text, pp. 882). The effect is to reduce the degrees of freedom for the various estimates of components of variance.

The 0.95 confidence limits for the residual variance were computed by the method described in most text books.[21]

Estimates of the 0.95 confidence limits for the other components of variance were derived by a method suggested in Owen L. Davies (Ed.), *Statistical Methods for Research and Production*, 3rd Ed., and discussed in A. J. Duncan, *Quality Control and Industrial Statistics*, Rev. Ed., 1959, pp. 623 ff. The method used in each case may be illustrated by showing how 0.95 confidence limits for the components of variance for available phosphorus in Fertilizer A were computed. The procedures were as follows:

0.95 Confidence Limits for the Standard Deviation of the Residual Variation. — Here we have $\chi^2_{0.975}$ for 18 degrees of freedom, symbolized by $\chi^2_{0.975}(18)$, equal to 8.23. Also $\chi^2_{0.025}(18) = 31.5$. Then 0.95 confidence limits for the residual variance are given by:

$$\frac{0.1056}{31.5} = 0.0034 \text{ and } \frac{0.1056}{8.23} = 0.0128,$$

and those for the standard deviation by:

$$\sqrt{0.0034} = 0.06 \text{ and } \sqrt{0.0128} = 0.11.$$

[20] Compare H. Leon Harter and Mary D. Lum, *Partially Hierarchal Models in the Analysis of Variance* (WADC Technical Report 55-33), pp. 53 ff.

[21] *See*, for example, A. J. Duncan, *Quality Control and Industrial Statistics*, Rev. Ed., 1959, pp. 433, 623.

Approximate 0.95 Confidence Limits for the Net Standard Deviation of the Interaction Variation Bags × Days. — Here we have $F_{0.025}(15,18) = 2.69$ and $F_{0.025}(18,15) = 2.80$. (Linear interpolation in the F table was deemed sufficiently accurate for our purposes.) Then 0.95 confidence limits for $(n_c\sigma_{bd}^2 + \sigma^2)/\sigma^2$ are given by:

$$\frac{0.0069}{(2.69)(0.0059)} \leq \frac{2\sigma_{bd}^2 + \sigma^2}{\sigma^2} \leq \frac{0.0069(2.80)}{0.0059}.$$

If we estimate σ^2 as equal to the residual mean square 0.0059, then approximate 0.95 confidence limits for σ_{bd}^2 will be given by:

$$\frac{0.0069 - 0.0059(2.69)}{2(2.69)} \leq \sigma_{bd}^2 \leq \frac{0.0069(2.80) - 0.0059}{2},$$

or by $0 \leq \sigma_{bd}^2 \leq 0.0067$, since σ_{bd}^2 cannot be negative. Hence approximate 0.95 confidence limits for σ_{bd} will be given by:

$$0 \leq \sigma_{bd} \leq 0.08$$

Approximate 0.95 Confidence Limits for the Net Standard Deviation of the Sample-to-Sample Variation. — Here $F_{0.025}(18,18) = 2.62$. Then approximate 0.95 confidence limits for σ^2 will be given by:

$$\frac{0.0120 - 0.0059(2.62)}{2(2.62)} \leq \sigma_e^2 \leq \frac{0.0120(2.62) - 0.0059}{2}, \text{ or}$$

$$0 \leq \sigma_e^2 \leq 0.0128$$

and approximate 0.95 confidence limits for σ_e will be given by:

$$0 \leq \sigma_e \leq 0.11$$

Approximate 0.95 Confidence Limits for the Net Standard Deviation of the Day-to-Day Variation. — Here $F_{0.025}(3,15) = 4.15$ and $F_{0.025}(15,3) = 14.3$. Then approximate 0.95 confidence limits for σ_d^2 will be given by:

$$\frac{0.0528 - 0.0069(4.15)}{12(4.15)} \leq \sigma_d^2 \leq \frac{0.0528(14.3) - 0.0069}{12},$$

or by:

$$0.0005 \leq \sigma_d^2 \leq 0.0623$$

and limits for σ_d by:

$$0.02 \leq \sigma_d \leq 0.25$$

Approximate 0.95 Confidence Limits for the Net Standard Deviation of the Bag-to-Bag Variation. — Here $F_{0.025}(15,18) = 2.69$ and $F_{0.025}(18,15) = 2.80$.

The 0.95 confidence limits for

$$\frac{n_c n_d \sigma^2 + n_d\sigma^2 + n_c\sigma_{bd}^2 + \sigma^2}{n_d\sigma_e^2 + \sigma^2}$$

will be given by:

$$\frac{1.0288}{2.69(0.0120)} \leq \frac{4\sigma_b^2 + 2\sigma_e^2 + 2\sigma_{bd}^2 + \sigma^2}{2\sigma_e^2 + \sigma^2} \leq \frac{1.0288(2.80)}{0.0120}$$

If we substitute the sample mean square (s_4^2) for $2\sigma_e^2 + \sigma^2$, approximate 0.95 confidence limits for $2\sigma_b^2 + \sigma_{bd}^2$ will be given by:

$$\frac{1.0288 - (0.0120)(2.69)}{2(2.69)} \leq 2\sigma^2 + \sigma_{bd}^2 \leq \frac{1.0288(2.80) - 0.0120}{2},$$

and on substituting the estimate of σ_{bd}^2, we get approximate 0.95 confidence limits for σ_b^2 as equal to:

$$0.0924 \leq \sigma_b^2 \leq 0.7169$$

and those for σ_b equal to:

$$0.30 \leq \sigma_b^2 \leq 0.85.$$

Approximate 0.95 Confidence Limits for the Net Standard Deviation of the Block-to-Block Variation. — Here $F_{0.025}(2,15) = 4.77$ and $F_{0.025}(15,2) = 39.4$. Then 0.95 confidence limits for

$$R = \frac{n_b n_c n_d \sigma_a^2 + n_c n_d \sigma_b^2 + n_d \sigma_e^2 + n_b n_c \sigma_d^2 + n_c \sigma_{bd}^2 + \sigma^2}{n_c n_d \sigma_b^2 + n_d \sigma_e^2 + n_c \sigma_{bd}^2 + \sigma^2}$$

will be given by:

$$\frac{0.2505}{1.028(4.77)} \leq R \leq \frac{0.2505(39.4)}{1.028}.$$

If the denominator of R is estimated as equal to the mean square for bag-to-bag variation (i.e. as equal to s_3^2), then approximate 0.95 confidence limits for $n_d\sigma_a^2 + \sigma_d^2$ will be given by:

$$\frac{0.2505 - 1.028(4.77)}{12(4.77)} \leq 2\sigma_a^2 + \sigma_d^2 \leq \frac{0.2505(39.4) - 1.0288}{12},$$

or by:

$$0 \leq 2\sigma_a^2 + \sigma_d^2 \leq 0.7367$$

and if the estimate of σ_d^2 computed above is used for σ_d^2, then approximate 0.95 confidence limits for σ_a^2 will be given by:

$$0 \leq \sigma_a^2 \leq 0.3665$$

Approximate 0.95 confidence limits for σ_a will be given by:

$$0 \leq \sigma_a \leq 0.61.$$

It will be noted from Table A-11 that in some cases an estimate of a component of

variance is based only on one degree of freedom. In these instances the upper confidence limit is generally so high as to be of little value.

Note 18. The Analysis of Variance of the Data of Steps 15, 30, and 45 and Estimates of Components of Variance

The procedure for this analysis is very similar to that described in Note 17 and will not be given in detail. The mathematical model is the same except that (*1*) the day-to-day variation becomes part of the block variation since all tests in a given block were run the same day, (*2*) any existent bag-day interaction becomes confounded with the bag-to-bag variation, and (*3*) the within-day laboratory test error becomes confounded with the sample-to-sample variation.

The expected values of the various mean squares are:

Symbol Used	*Mean Square*	*Expected Value*
s_1^2	Bet. Blocks	$n_b n_c \sigma_a^2 + n_c \sigma_b^2 + \sigma^2$
s_2^2	Bet. Bags *w.* Blocks	$n_c \sigma_b^2 + \sigma^2$
s_3^2	Bet. Samples *w.* Bags	σ^2

where n_b is the number of bags (*viz.*, 8), n_c is the number of samples (riffles) per bag (*viz.*, 2), σ_a^2 is the new block variance, σ^2 the new bag-to-bag variance, and σ^2 is the combined variance of sample-to-sample variation and laboratory variation (within the same day).

An estimate of σ_b^2 is thus given by:

$$\text{Est. of } \sigma_b^2 = \frac{s_2^2 - s_3^2}{2},$$

and an estimate of σ_a^2 is given by:

$$\text{Estimate of } \sigma_a^2 = \frac{s_1^2 - s_2^2}{16}.$$

Confidence limits are obtained in a manner similar to that described in Note 17.

Note 19. Computation of the Net Components of Variance of Table VI-4

The tolerances of Table VI-4 were derived from the sum of net components. These were in turn derived from the mean squares[22] presented in Table A-11 and other tables. The details of the procedure are given in Table A-13. The various mean squares employed are all unbiased estimates of particular functions of the net components of variance and the over-all variance is a linear function of these mean squares.

Confidence limits for the tolerances were not generally computed. Approximate limits may be calculated, however, with the help of F. E. Satterthwaite's approximation. This says that if some complex variance σ^2 is estimated by:

$$L = \sum_i k_i s_i^2$$

where the k_i are varying constants and the s_i^2 are mean squares with n_i degrees of freedom respectively, then $\frac{mL}{\sigma^2}$ is approximately distributed as χ^2 with m degrees of freedom where m is given by:[23]

$$m = \frac{[\sum k_i E(s_i^2)]^2}{\sum \frac{[k_i E(s_i^2)]^2}{n_i}}$$

In practice we usually use the s_i^2 as estimates of $E(s_i^2)$.

To illustrate how this approximation may be used, let us compute confidence limits for the tolerance for total phosphorus of Fertilizer A. Let the following s_i^2 be taken from Table A-11:

s_2^2 = mean square for days within blocks
s_3^2 = mean square for bags within blocks
s_4^2 = mean square for samples within bags
s_5^2 = bags × days within blocks interaction mean square
s_6^2 = residual mean square

and let the following mean squares be taken from Table A-9:

$s_L'^2$ = mean square for laboratories divided by 100 (to decode)
$s_{L\times B}'^2$ = lab × bag interaction mean square divided by 100

The degrees of freedom for each of these mean squares will be:

[22] In one instance the net components of variance were derived from average ranges. This was based on the use of Patnaik's approximation which assumes that $(R/d_2^*)^2$ has approximately the same distribution as a mean square. *See* A. J. Duncan, Quality Control and Industrial Statistics, 2nd Ed., p. 117.

[23] *See* F. E. Satterthwaite, "Synthesis of Variance," *Psychometrika*, Vol. VI (1941) pp. 309-16, and "An Approximate Distribution of Estimates of Variance Components," *Biometrics*, Vol. II (1946), pp. 110-14.

for s_2^2, d.f. = 3
for s_3^2, d.f. = 18
for s_4^2, d.f. = 21
for s_5^2, d.f. = 18
for s_6^2, d.f. = 21
for $s_L'^2$, d.f. = 2
for $s_L'^2$ d.f. = 4

The procedure for calculating the total variance for use in determining the tolerance for total phosphorus of Fertilizer A as described in Table A-13, yields the final net formula[24] for this total tolerance:

$$s^2 = \frac{60s_2^2 + 21s_3^2 + 21s_4^2 - 81s_5^2 + 819s_6^2 + 280s_L'^2 - 280s_{L\times B}'^2}{1680}$$

The degrees of freedom with which this will be distributed are approximately:

$$\frac{s^4}{\frac{1}{(1680)^2}\left[\frac{(60s_2^2)^2}{3} + \frac{(21s_3^2)^2}{18} + \frac{(21s_4^2)^2}{21} + \frac{(81s_5^2)^2}{18} + \frac{(819s_6^2)^2}{21} + \frac{(280s_L'^2)^2}{2} + \frac{(280s_{L\times B}'^2)^2}{4}\right]}$$

For the values of the various s_i^2 given in Tables A-9 and A-11, the degrees of freedom for s^2 turns out to be approximately 4. The degrees of freedom are very small in this particular case since the total variance is dominated by the laboratory variance which is based on only 2 degrees of freedom. The formula should yield a larger number in other instances. Four degrees of freedom for the total variance means that 0.95 confidence limits for the tolerance for phosphorus of Fertilizer A will be given by:

$$2.326\sqrt{\frac{4s^2}{\chi^2_{.025}(4)}} \quad \text{and} \quad 2.326\sqrt{\frac{4s^2}{\chi^2_{.975}(4)}}$$

or by

$$2.326\sqrt{\frac{4(0.0443)}{11.1}} = 0.29$$

$$\text{and } 2.326\sqrt{\frac{4(0.0443)}{0.484}} = 1.41$$

or approximately 59% and 288% of the tolerance given in Table A-13. Since the degrees of freedom depend in each case on the values of the mean squares, separate confidence limits will have to be computed for each case. The above is at least an example of how wide the confidence limits may be.

[24] Note that in this case the net variance component for riffling is taken as zero.

APPENDIX TABLES

Table A-1. Analysis of variance of sieve weights for Fertilizers A and D with order of sampling viewed as a source of variation (Units in per cent)

Fertilizer and Sieve Size	SS					ms					F-Ratio			
	Inst.	Men	Order	Blocks	Error	Inst.	Men	Order	Blocks	Error	Inst.	Men	Order	Blocks
A: 10-10-10 Granulated														
Mesh 6	34.17	7.22	1.87	72.36	30.78	17.09	3.61	0.94	36.18	1.71	9.99**	2.11	0.55	21.16**
Mesh 14	16.93	52.43	45.09	57.22	178.60	8.47	26.22	22.55	28.61	9.92	0.85	2.64	2.27	2.88
Mesh 24	33.02	28.39	2.48	178.59	95.94	16.51	14.20	1.24	89.30	5.33	3.10	2.66	0.23	16.75**
D: 8-16-16 granulated														
Mesh 6	36.51	1.29	3.84	391.51	55.66	18.26	0.65	1.92	195.76	3.09	5.91*	0.21	0.62	63.35**
Mesh 14	3.08	41.72	22.21	1889.36	206.77	1.54	20.86	11.11	944.68	11.49	0.13	1.82	0.97	82.22**
Mesh 24	7.83	3.42	14.13	368.58	45.61	3.92	1.71	7.07	184.29	2.53	1.55	0.68	2.79	72.84**
df	2	2	2	2	18									
Critical F values[a] 5%													3.57	
Critical F values[a] 1%													6.05	

[a] Obtained by linear interpolations.
* Results significant at the 0.05 level.
** Results significant at the 0.01 level or lower.

Table A-2. Analysis of variance of chemical determinations with order of sampling viewed as a source of variation (Units in 0.1%)

Source of Variation	SS					ms					F-Ratio			
	Inst.	Men	Order	Blocks	Error	Inst.	Men	Order	Blocks	Error	Inst.	Men	Order	Blocks
A: 10-10-10 granulated														
N	0.22	2.89	0.89	460.22	38.45	0.11	1.45	0.45	230.11	2.14	0.05	0.68	0.21	107.53**
TP	0.96	7.19	2.29	93.41	18.22	0.48	3.60	1.15	46.71	1.01	0.48	3.56	1.14	46.25**
AP	0.52	6.74	1.41	128.30	12.22	0.26	3.37	0.72	64.15	0.68	0.38	4.96*	1.04	94.34**
K	1.41	15.63	21.63	5.63	56.66	0.71	7.82	10.82	2.82	3.15	0.23	2.48	3.43	0.90
B: 0-20-20 powdered														
TP	10.96	9.41	25.85	73.85	216.89	5.48	4.71	12.93	36.93	12.05	0.45	0.39	1.07	3.06
AP	11.19	8.07	26.96	156.07	198.45	5.60	4.04	13.48	78.04	11.03	0.51	0.37	1.22	7.08**
K	7.19	15.63	4.74	1370.96	152.00	3.60	7.82	2.37	685.48	8.44	0.43	0.93	0.28	81.22**
C: 5-10-10 powdered														
N	3.11	8.11	2.11	272.22	6.23	1.56	4.06	1.06	272.22	0.62	2.52	6.55**	1.71	439.06**
TP	4.11	1.44	1.44	43.56	5.89	2.06	0.72	0.72	43.56	0.59	3.49	1.22	1.22	73.83**
AP	4.78	3.44	0.78	174.23	7.22	2.39	1.72	0.39	174.23	0.72	3.18	2.34	0.54	241.99**
K	0.11	5.44	0.11	234.72	2.56	0.06	2.72	0.06	234.72	0.26	0.23	10.46**	0.23	902.77**
D: 8-16-16 granulated														
N	29.41	9.85	14.30	656.96	94.89	14.71	4.93	7.15	328.48	5.27	2.79	0.94	1.36	62.33**
TP	25.41	5.41	1.85	644.96	74.44	12.71	2.71	0.93	322.48	4.14	3.07	0.65	0.22	77.89**
AP	22.74	4.74	3.63	678.30	71.11	11.37	2.37	1.82	339.15	3.95	2.88	0.60	0.46	85.86**
K	32.89	8.67	20.22	1243.56	67.33	16.45	4.34	10.11	621.78	3.74	4.40*	1.16	2.70	166.25**
df	2	2	2	2	18									
Critical F values[a] 5%													3.57	
Critical F values[a] 1%													6.05	

[a] Obtained by linear interpolation.
* Results significant at the 0.05 level.
** Results significant at the 0.01 level or lower.

Table A-3. Analysis of variance of sieve weights for A and D Fertilizers with order of sampling randomized (Units in per cent)

Fertilizer and Sieve Size	ms							F-Ratio					
	Inst.	Men	Blocks	Inst. × Men	Inst. × Blocks	Men × Blocks	Error	Inst.	Men	Blocks	Inst. × Men	Inst. × Blocks	Men × Blocks
A: 10-10-10, granulated													
Mesh 6	21.56	2.47	44.88	1.39	0.57	4.03	1.57	13.73**	1.57	28.58**	0.88	0.36	2.57
Mesh 14	3.08	42.81	43.14	17.83	5.19	7.33	7.83	0.39	5.47*	5.51*	2.28	0.66	0.94
Mesh 24	21.38	16.75	107.90	6.36	2.25	5.46	4.15	5.16*	4.04*	26.03**	1.53	0.54	1.32
D: 8-16-16, granulated													
Mesh 6	16.11	0.46	237.41	3.53	4.09	5.75	3.35	4.81*	0.14	70.87**	1.05	1.22	1.72
Mesh 14	6.25	14.09	1022.31	6.13	17.53	30.68	11.79	0.53	1.20	86.71**	0.52	1.49	2.60
Mesh 24	4.51	2.05	201.84	0.77	2.36	5.82	5.30	0.85	0.39	38.08**	0.15	0.45	1.10
df	2	3	2	6	4	6	12						
Critical F values 5%								3.89	3.49	3.89	3.00	3.26	3.00
Critical F values 1%								6.93	5.95	6.93	4.82	5.41	4.82

* Results significant at the 0.05 level.
** Results significant at the 0.01 level or lower.

Table A-4. Analysis of variance of chemical determinations with order of sampling randomized (Units in 0.1%)

Source of Variation	ms							F-Ratio					
	Inst.	Men	Block	Men and Inst.	Inst. and Block	Men and Block	Residual Error	Inst.	Men	Block	Men and Inst.	Inst. and Block	Men and Block
Fert. *A:* 10-10-10, granulated													
N	0.36	1.66	305.36	2.10	1.07	2.44	1.26	0.29	1.32	243.31	1.67	0.85	1.94
TP	0.36	5.36	63.20	0.70	0.53	1.86	0.53	0.68	10.11**	119.25**	1.32	1.00	3.51*
AP	0.36	3.74	80.11	0.55	0.36	1.19	0.32	1.13	11.69**	250.34**	1.71	1.13	3.71*
K	0.36	5.58	3.86	3.49	1.90	3.65	2.90	0.12	1.92	1.33	1.20	0.66	1.26
Fert. *B:* 0-20-20, powdered													
TP	13.78	12.47	70.20	3.33	2.19	21.19	10.14	1.36	1.23	6.92*	0.33	0.22	2.09
AP	13.59	12.37	129.09	2.95	1.67	17.90	10.82	1.26	1.14	11.93**	0.27	0.15	1.65
K	8.70	6.44	1018.70	4.70	9.61	17.81	3.06	2.84	2.10	332.91**	1.54	3.14	5.82**
Fert. *C:* 5-10-10, powdered													
N	0.78	1.11	592.70	0.78	0.49	1.47	0.43	1.81	2.58	378.37**	1.81	1.14	3.42*
TP	1.00	0.19	91.00	0.63	0.50	1.07	1.02	0.98	0.19	89.22**	0.62	0.49	1.05
AP	1.70	0.18	142.20	1.07	0.82	1.90	1.08	1.57	0.16	131.67**	0.99	0.76	1.76
K	8.45	3.26	198.86	2.59	5.11	0.57	3.31	2.55	0.98	60.08**	0.78	1.54	0.17
Fert. *D:* 8-16-16, granulated													
N	24.09	4.07	480.59	2.49	3.54	5.55	4.67	5.16*	0.87	102.91**	0.53	0.76	1.19
TP	9.70	4.96	412.03	4.33	8.03	9.32	4.10	2.37	1.21	100.50**	1.06	1.96	2.27
AP	7.75	5.81	419.25	4.75	6.63	9.81	2.96	2.62	1.96	141.64**	1.60	2.24	3.31*
K	23.09	4.25	651.00	4.86	8.08	17.45	9.31	2.48	0.46	69.92**	0.52	0.87	1.87
df	2	3	2	6	4	6	12						
Critical F Values 5%								3.89	3.49	3.89	3.00	3.26	3.00
Critical F Values 1%								6.93	5.95	6.93	4.82	5.41	4.82

* Results significant at the 0.05 level.
** Results significant at the 0.01 level or lower.

Table A-5. Means of two instrument samples and two riffles from same bags

(Data in per cent. First week only)

Block 1			Block 2			Block 3		
Bag	Instr.	Riffle	Bag	Instr.	Riffle	Bag	Instr.	Riffle
FERTILIZER A: 10–10–10, Granulated								
				Nitrogen				
2	10.35	10.50	10	9.70	9.95	18	9.72	9.85
3	10.10	10.30	11	9.78	9.84	19	9.78	9.72
4	9.60	10.20	12	9.78	9.78	20	9.90	9.75
5	10.70	10.15	13	9.84	9.74	21	9.92	9.85
6	10.50	10.35	14	9.83	9.88	22	9.40	9.48
7	10.45	10.25	15	9.74	9.76	23	9.75	9.45
8	10.40	10.45	16	9.60	9.72	24	9.68	9.60
Means	10.30	10.31		9.75	9.81		9.74	9.64
				Total Phosphorus (P_2O_5)				
2	10.95	11.00	10	11.10	11.18	18	10.85	10.92
3	11.25	11.15	11	11.12	11.16	19	10.95	10.85
4	13.10	12.90	12	11.22	11.42	20	10.98	10.98
5	11.10	11.15	13	11.08	11.11	21	10.90	11.00
6	11.05	11.10	14	11.04	11.10	22	11.92	12.10
7	11.15	11.15	15	11.17	11.10	23	11.48	11.32
8	11.20	11.25	16	11.54	11.54	24	11.05	10.98
Means	11.40	11.38		11.18	11.23		11.16	11.16
				Available Phosphorus (P_2O_5)				
2	10.72	10.82	10	10.96	11.10	18	10.70	10.78
3	11.05	11.00	11	10.98	11.04	19	10.78	10.70
4	12.75	12.58	12	11.08	11.23	20	10.85	10.88
5	10.85	10.92	13	10.95	11.01	21	10.78	10.85
6	10.88	10.92	14	10.94	10.98	22	11.72	11.85
7	10.95	10.88	15	11.05	10.98	23	11.28	11.10
8	11.02	11.10	16	11.40	11.38	24	10.92	10.82
Means	11.17	11.17		11.05	11.10		11.00	11.00
				Potash				
2	10.85	10.70	10	10.56	10.60	18	10.60	10.60
3	11.02	10.95	11	10.54	10.54	19	10.84	10.32
4	12.88	12.55	12	10.77	10.93	20	10.77	10.78
5	10.70	10.88	13	10.54	10.58	21	10.90	11.56
6	10.85	10.78	14	10.67	10.65	22	12.15	11.32
7	10.62	10.70	15	10.94	10.82	23	11.58	11.18
8	10.50	10.58	16	11.38	11.32	24	10.78	10.76
Means	11.06	11.02		10.77	10.78		11.09	10.93
FERTILIZER B: 0–20–20, Powdered								
				Total Phosphorus (P_2O_5)				
26	20.55	20.50	34	20.27	19.52	42	23.50	22.68
27	20.70	20.80	35	21.96	21.30	43	20.82	20.45
28	21.70	21.60	36	20.73	20.91	44	20.65	20.48
29	20.05	20.35	37	20.69	20.86	45	20.05	20.58
30	21.00	21.40	38	21.52	19.94	46	20.85	21.15
31	22.00	21.75	39	21.34	20.81	47	21.15	21.80
32	20.60	20.80	40	21.73	20.40	48	20.82	21.20
Means	20.94	21.03		21.18	20.53		21.12	21.19

(Continued)

Table A-5 (*Continued*)

Block 1			Block 2			Block 3		
Bag	Instr.	Riffle	Bag	Instr.	Riffle	Bag	Instr.	Riffle
FERTILIZER B: 0–20–20, Powdered								
			Available Phosphorus (P_2O_5)					
26	19.78	19.70	34	19.79	18.94	42	22.60	21.80
27	20.12	20.20	35	21.40	20.77	43	19.95	19.72
28	20.88	20.85	36	20.25	20.44	44	19.98	19.82
29	19.38	19.60	37	20.33	20.42	45	19.35	19.95
30	20.22	20.58	38	21.05	19.44	46	20.05	20.42
31	21.22	20.95	39	20.83	20.32	47	20.58	21.20
32	19.82	20.02	40	21.23	19.88	48	20.12	20.48
Means	20.20	20.27		20.70	20.03		20.38	20.48
			Potash					
26	22.25	21.95	34	18.84	19.56	42	17.67	17.86
27	20.95	20.40	35	20.24	20.40	43	19.68	19.85
28	20.80	20.25	36	21.67	20.23	44	20.76	20.54
29	22.25	21.85	37	20.70	19.72	45	20.73	19.60
30	21.20	20.80	38	21.08	21.01	46	20.59	20.80
31	19.85	18.80	39	21.15	20.26	47	21.04	20.12
32	21.05	20.70	40	20.70	21.33	48	21.38	21.04
Means	21.19	20.68		20.63	20.36		20.26	19.97
FERTILIZER C: 5–10–10, Powdered								
			Nitrogen					
50	5.45	5.42	58	5.50	5.30	66	5.35	5.20
51	5.48	5.45	59	5.22	5.20	67	5.30	5.22
52	5.30	5.40	60	5.55	5.33	68	5.30	5.22
53	5.35	5.35	61	5.35	5.22	69	5.35	5.28
54	5.50	5.35	62	5.50	5.38	70	5.28	5.35
55	5.50	5.38	63	5.34	5.31	71	5.32	5.22
56	5.52	5.65	64	5.35	5.29	72	5.30	5.20
Means	5.44	5.43		5.40	5.29		5.31	5.24
			Total Phosphorus (P_2O_5)					
50	11.05	10.85	58	10.86	10.74	66	10.78	10.78
51	11.00	11.00	59	11.21	11.25	67	10.90	10.88
52	11.35	10.90	60	10.80	10.82	68	11.08	11.10
53	11.00	10.90	61	10.95	10.94	69	10.50	10.70
54	10.75	10.90	62	10.62	10.58	70	10.88	10.95
55	11.10	10.85	63	11.00	10.72	71	11.12	10.95
56	10.70	10.55	64	10.86	10.90	72	11.12	10.90
Means	10.99	10.85		10.90	10.85		10.91	10.89
			Available Phosphorus (P_2O_5)					
50	10.20	10.00	58	10.23	10.04	66	10.02	9.92
51	10.10	10.08	59	10.58	10.45	67	10.12	10.05
52	10.50	10.05	60	10.10	10.10	68	10.28	10.18
53	10.15	10.05	61	10.24	10.26	69	9.75	9.80
54	9.88	10.02	62	10.00	10.06	70	10.02	10.10
55	10.18	9.95	63	10.34	10.02	71	10.35	10.12
56	9.85	9.72	64	10.21	10.26	72	10.33	10.10
Means	10.12	9.98		10.24	10.17		10.12	10.04

Table A-5 (*Continued*)

Block 1			Block 2			Block 3		
Bag	Instr.	Riffle	Bag	Instr.	Riffle	Bag	Instr.	Riffle
FERTILIZER C: 5–10–10, Powdered								
				Potash				
50	10.80	10.68	58	11.00	10.90	66	11.06	10.82
51	10.70	10.88	59	10.84	10.92	67	11.26	10.94
52	11.05	10.90	60	11.02	10.90	68	10.94	10.68
53	11.35	11.28	61	10.97	10.90	69	11.36	11.04
54	11.00	10.90	62	11.33	11.06	70	11.06	10.78
55	11.08	10.95	63	11.03	10.88	71	11.22	10.97
56	11.10	10.90	64	10.98	10.83	72	10.94	10.90
Means	11.01	10.93		11.02	10.91		11.12	10.88
FERTILIZER D: 8–16–16, Granulated								
				Nitrogen				
74	7.70	7.95	82	7.69	7.78	90	7.80	7.98
75	8.35	8.55	83	7.84	7.84	91	7.90	8.00
76	8.40	8.40	84	7.80	7.86	92	7.75	7.62
77	8.40	8.30	85	7.76	7.88	93	7.78	7.70
78	8.45	8.30	86	7.51	7.52	94	7.85	7.82
79	8.25	8.35	87	7.76	7.80	95	7.75	7.85
80	8.30	8.35	88	7.98	7.98	96	7.45	7.58
Means	8.26	8.31		7.76	7.81		7.75	7.79
				Total Phosphorus (P_2O_5)				
74	15.90	15.85	82	17.34	17.44	90	17.40	17.68
75	17.50	17.75	83	17.56	17.54	91	17.78	17.58
76	17.80	18.20	84	17.72	17.79	92	16.58	16.35
77	17.60	17.80	85	17.97	18.14	93	17.48	17.08
78	17.35	17.80	86	17.48	17.52	94	17.35	17.52
79	17.10	17.30	87	17.56	17.78	95	17.60	17.35
80	17.40	17.75	88	18.10	18.20	96	17.70	17.45
Means	17.24	17.49		17.38	17.77		17.41	17.29
				Available Phosphorus (P_2O_5)				
74	15.40	15.38	82	16.86	17.00	90	16.78	17.12
75	16.88	17.12	83	17.10	17.12	91	17.18	16.98
76	17.15	17.58	84	17.25	17.31	92	16.02	15.90
77	17.00	17.18	85	17.50	17.71	93	16.88	16.45
78	16.70	17.18	86	17.02	17.14	94	16.75	16.98
79	16.48	16.70	87	17.06	17.36	95	17.00	16.80
80	16.80	17.15	88	17.59	17.76	96	17.05	16.85
Means	16.63	16.90		17.20	17.34		16.81	16.72
				Potash				
74	15.85	15.30	82	17.62	17.76	90	16.87	16.17
75	17.00	16.08	83	17.50	17.54	91	16.75	16.12
76	16.15	15.65	84	16.99	16.82	92	15.62	15.10
77	16.85	16.12	85	16.17	16.08	93	17.54	17.33
78	16.88	16.22	86	17.94	17.60	94	17.06	16.52
79	17.75	17.42	87	17.22	17.35	95	17.24	16.70
80	17.25	16.35	88	16.26	15.91	96	16.69	16.70
Means	16.82	16.16		17.10	17.01		16.82	16.38

Table A-6. Analysis of variance of instrument sampling vs. riffling
(Original data, totals of two samples in per cent. First week's tests only)

Fertilizer and Chemical Element	Mean Squares					F-Ratio		
	Type of Sampling	Blocks	Bags *w.* Blocks	Blk. × Type	Residual	Type / Res.	Blk. / Bags	Blk. × T / Res.
A. 10–10–10								
N	0.0069	5.1235	0.2451	0.0049	0.0920	0.08	20.9**	0.05
TP	0.0067	0.8377	1.8909	0.0147	0.0205	0.33	0.44	0.72
AP	0.0074	0.4308	1.6159	0.0122	0.0195	0.38	0.27	0.63
K	0.1672	1.1795	2.2441	0.1013	0.1789	0.93	0.52	0.57
B. 0–20–20								
TP	1.1172	1.2643	4.0413	2.4180	0.5316	2.10	0.31	4.55*
AP	1.1075	0.5364	3.6912	2.6665	0.5164	2.14	0.15	5.16*
K	5.4432	9.3665	6.9814	0.2510	0.7244	7.51*	1.34	0.35
C. 5–10–10								
N	0.1827	0.3480	0.0379	0.0339	0.0144	12.69**	9.18**	2.35
TP	0.1934	0.0292	0.2348	0.1552	0.0541	3.57	0.12	2.81
AP	0.5929	0.3296	0.2427	0.0212	0.0760	7.80*	1.36	0.28
K	1.1372	0.0154	0.1710	0.0058	0.0198	59.19**	0.09	0.29
D. 6–18–18								
N	0.0851	4.8606	0.2270	0.0004	0.0245	3.47	21.41**	0.02
TP	0.2479	2.5369	1.9622	0.5163	0.0641	3.89	1.29	8.05**
AP	0.5192	4.7540	1.6994	0.4429	0.1296	4.01	2.80	3.42
K	6.3726	4.7625	3.4833	1.2318	0.0971	65.63**	1.37	12.69**
df	1	2	18	2	18			
Critical F values[a] 5%						4.43	3.57	3.57
Critical F values[a] 1%						8.33	6.05	6.05

[a] Obtained by linear interpolation. * Results significant at the 0.05 level.
** Results significant at the 0.01 level or lower.

Table A-7. Further analysis of variance of instrument sampling vs. riffling: Fertilizer A
(Original data totals of two samples in per cent. Second week's tests)

Chemical Element	Mean Squares					F-Ratios		
	Type	Block	Bag/Bl.	Bl. × T	Residual	Type / Res.	Block / Bag/Bl.	Bl. × T / Res.
N	0.0721	4.5528	0.1942	0.1638	0.0636	1.13	23.44**	2.58
TP	0.0003	0.2189	1.9677	0.1622	0.0229	0.01	0.11	7.08**
AP	0.0016	0.2306	1.6782	0.1344	0.0201	0.08	0.14	6.69**
K	0.1989	0.9547	2.1365	0.0163	0.0808	2.46	0.45	0.20
df	1	2	18	2	18			
Critical F values[a] 5%						4.43	3.57	3.57
Critical F values[a] 1%						8.33	6.05	6.05

[a] Obtained by linear interpolation. ** Results significant at the 0.01 level or lower.

Table A-8. Analysis of variance of absolute within-bag sample-to-sample differences: Instrument sampling vs. riffling

(Data in per cent. First week only)

Fertilizer and Chemical Element	Mean Squares					F-Ratio		
	Type of Sampling	Blocks	Bags *w.* Blocks	Blk. × Type	Residual	Type / Res.	Blk. / Bags	Blk. × T / Res.
A. 10–10–10								
N	0.0092	0.0492	0.0102	0.0119	0.0161	0.03	4.81*	0.74
TP	0.0088	0.0024	0.0027	0.0064	0.0046	1.91	0.89	1.39
AP	0.0018	0.0083	0.0036	0.0069	0.0059	0.31	0.23	1.17
K	0.0148	0.0703	0.0122	0.0177	0.0210	0.70	5.76*	0.84
B. 0–20–20								
TP	0.1040	0.1107	0.0698	0.4229	0.0659	1.58	1.59	6.42**
AP	0.1238	0.1255	0.0498	0.3547	0.0673	1.84	2.52	5.27*
K	0.0123	1.4720	0.0934	0.1047	0.0960	0.13	15.76**	1.09
C. 5–10–10								
N	0.0009	0.0379	0.0046	0.0185	0.0060	0.15	8.24**	3.08
TP	0.0176	0.0297	0.0205	0.0722	0.0266	0.66	1.45	2.71
AP	0.0224	0.0282	0.0206	0.0538	0.0238	0.94	1.37	2.26
K	0.0156	0.0001	0.0121	0.0119	0.0129	1.21	0.01	0.92
D. 8–16–16								
N	0.0315	0.0569	0.0191	0.0243	0.0123	2.56	2.98	1.98
TP	0.0005	0.0894	0.0261	0.0102	0.0202	0.02	3.43	0.50
AP	0.0017	0.0616	0.0200	0.0225	0.0196	0.09	3.08	1.24
K	0.0083	0.1574	0.0378	0.1391	0.0486	0.17	4.16*	2.86
df	1	2	18	2	18			
Critical F values[a]					5%	4.43	3.57	3.57
					1%	8.33	6.05	6.05

[a] Obtained by linear interpolation.
* Results significant at the 0.05 level.
** Results significant at the 0.01 level or lower.

Table A-9. Analyses of variance of interlaboratory comparisons

(Data measured in 10ths of one per cent)

Fertilizer and Chemical Element	Mean Squares				F-Ratio		
	Bag	Lab	L × B	Error	L × B	Bag	Lab
A. 10–10–10, granulated							
N	41.72	59.72	3.06	3.78	0.81	11.04**	19.52**
Total P_2O_5	72.06	19.06	4.55	0.72	6.32*	100.08**	4.19
Insoluble P_2O_5	0.18	0.94	0.48	0.43	1.12	0.43	1.97
Available P_2O_5	63.17	30.50	2.17	1.11	1.95	56.91**	14.06*
K	15.06	0.39	2.06	0.44	4.70*	34.23**	0.18

(Continued)

Table A-9 (*Continued*)

Fertilizer and Chemical Element	Mean Squares				F-Ratio		
	Bag	Lab	L × B	Error	L × B	Bag	Lab
B. 0–20–20, powdered							
Total P_2O_5	96.50	20.67	4.67	3.06	1.52	31.54**	4.43
Insoluble P_2O_5	2.18	17.10	2.21	0.22	10.00**	9.88**	7.74*
Available P_2O_5	109.06	52.72	9.31	3.00	3.10	36.35**	5.66
K	568.39	2.06	5.06	5.17	0.98	109.94**	0.41
C. 5–10–10, powdered							
N	156.72	8.72	0.89	0.22	4.05*	712.36**	9.80*
Total P_2O_5	80.17	15.17	1.34	0.67	2.00	119.66**	11.32*
Insoluble P_2O_5	6.35	9.05	2.09	0.22	9.71**	29.54**	4.33
Available P_2O_5	56.17	23.17	5.34	1.17	4.56*	48.01**	4.34
K	109.06	12.39	19.89	2.00	9.95**	54.53**	0.62
D. 8–16–16, granulated							
N	10.50	50.00	0.50	0.83	0.60	12.65**	100.00**
Total P_2O_5	211.72	4.22	3.39	1.00	3.39	211.72**	1.24
Insoluble P_2O_5	0.22	2.29	3.38	0.10	33.80**	2.29	0.68
Available P_2O_5	200.06	8.39	9.72	1.11	8.76**	180.23**	0.86
K	257.06	9.73	4.06	1.17	3.47	219.71**	2.40
df	2	2	2	9			
Critical F values[a]				5%	3.63	4.26	6.94
				1%	6.42	8.02	18.0

[a] Obtained by linear interpolation.
* Results significant at the 0.05 level.
** Results significant at the 0.01 level or lower.

Table A-10. Bagging variations[a]

Order of Bag Drawn	Bag Marking	Date	Experimental Bag Number	Lab.	Chemical Elements			
					TP	AP	N	K
Fertilizer A: 10–10–10, Granulated								
1	008	26th	12	2	11.42	11.23	9.78	10.93
2	011	26th	13	2	11.11	11.01	9.74	10.59
4	021	26th	8	1	11.25	11.10	10.45	10.58
6	024	27th	1	1	11.20	11.03	10.30	10.68
7	032	27th	3	1	11.15	11.00	10.30	10.95
8	041	27th	6	1	11.10	10.93	10.35	10.78
10	061	28th	20	3	10.98	10.88	9.75	10.78
15	076	28th	23	3	11.33	11.10	9.45	11.18
16	077	28th	16	2	11.55	11.38	9.72	11.33
17	084	29th	18	3	10.93	10.78	9.85	10.61
18	087	29th	5	1	11.15	10.93	10.15	10.88
19	094	29th	11	2	11.16	11.04	9.84	10.55
22	104	30th	14	2	11.10	10.98	9.89	10.65

(Continued)

Table A-10 (*Continued*)

Order of Bag Drawn	Bag Marking	Date	Experimental Bag Number	Lab.	Chemical Elements			
					TP	AP	N	K
23	111	30th	10	2	11.19	11.10	9.95	10.60
24	124	3rd	19	3	10.85	10.70	9.73	10.32
26	126	3rd	7	1	11.15	10.88	10.25	10.70
27	127	3rd	15	2	11.10	10.98	9.76	10.83
28	131	3rd	21	3	11.00	10.85	9.85	11.56
29	133	3rd	24	3	10.98	10.83	9.60	10.77
32	156	5th	17	3	10.93	10.80	9.88	10.65
34	160, 2–3	5th	2	1	11.00	10.83	10.50	10.70
35	163	5th	9	2	11.54	11.38	9.45	10.93
36	164	6th	22	3	12.10	11.85	9.48	11.32
37	207	7th	4	1	12.90	12.58	10.20	12.55
			Fertilizer B: 0–20–20, Powdered					
3	001	22nd	42	3	22.68	21.80		17.87
5	003, 5–1	22nd	35	2	21.30	20.77		20.41
6	003, 5–6	22nd	46	3	21.15	20.43		20.80
10	018	23rd	30	1	21.40	20.58		20.80
11	021	23rd	26	1	20.50	19.70		21.95
16	034	26th	25	1	20.65	19.85		20.70
17	038	26th	33	2	22.05	21.48		20.15
19	053	28th	28	1	21.60	20.85		20.25
21	067	28th	43	3	20.45	19.73		19.85
23	081	28th	48	3	21.20	20.48		21.04
24	088	30th	40	2	20.40	19.88		21.33
25	092	30th	45	3	20.58	19.95		19.60
26	093	30th	34	2	19.52	18.94		19.56
27	095	30th	44	3	20.48	19.83		20.54
28	103	30th	31	1	21.75	20.95		18.80
29	110	30th	29	1	20.35	19.60		21.85
31	116	3rd	32	1	20.80	20.03		20.70
33	137, 5–3	4th	38	2	19.94	19.44		21.01
37	163, 1–1	5th	36	2	20.91	20.44		20.23
38	171	5th	39	2	20.81	20.32		20.27
41	203	12th	37	2	20.86	20.42		19.72
42	204	12th	47	3	21.80	21.20		20.13
45	223	16th	27	1	20.80	20.20		20.40
46	239	16th	41	3	21.40	20.80		19.07
			Fertilizer C: 5–10–10, Powdered					
6	020	22nd	57	2	11.245	10.655	6.02	11.56
7	029, 2–2	22nd	54	1	10.90	10.025	5.35	10.90
8	029	22nd	56	1	10.55	9.725	5.65	10.90
9	030	23rd	65	3	11.675	10.825	4.80	11.485
11	034	26th	69	3	10.70	9.80	5.275	11.04
14	047	26th	58	2	10.745	10.035	5.30	10.905
17	060	27th	59	2	11.25	10.45	5.205	10.925
19	088	27th	51	1	11.00	10.075	5.45	10.875
23	122	28th	61	2	10.935	10.265	5.215	10.905
25	127, 1–2	28th	50	1	10.85	10.00	5.425	10.675
26	127, 5–5	28th	70	3	10.95	10.10	5.35	10.775

(*Continued*)

Table A-10 (*Continued*)

Order of Bag Drawn	Bag Marking	Date	Experimental Bag Number	Lab.	Chemical Elements			
					TP	AP	N	K
27	129	28th	60	2	10.815	10.095	5.33	10.895
28	134	28th	52	1	11.40	10.05	5.40	10.90
30	145	30th	53	1	10.90	10.05	5.35	11.275
33	160	30th	55	1	10.85	9.95	5.375	10.95
34	161	30th	63	2	10.715	10.015	5.31	10.88
36	180	3rd	71	3	10.95	10.125	5.225	10.97
37	184	3rd	49	1	10.80	9.90	5.425	10.925
38	186	3rd	68	3	11.10	10.175	5.225	10.685
40	209	4th	64	2	10.895	10.265	5.29	10.83
41	217	4th	66	3	10.775	9.925	5.20	10.82
42	221	4th	62	2	10.585	10.055	5.38	11.065
44	229	4th	67	3	10.875	10.05	5.225	10.945
48	242	4th	72	3	10.90	10.10	5.20	10.905
			Fertilizer D: 8–16–16, Granulated					
1	007	26th	86	2	17.525	17.135	7.525	17.595
2	008	26th	83	2	17.545	17.125	7.84	17.54
3	015	26th	80	1	17.75	17.15	8.35	16.35
4	016	26th	93	3	17.075	16.45	7.70	17.33
7	022	26th	82	2	17.44	17.00	7.775	17.765
8	024	26th	91	3	17.575	16.975	8.00	16.12
10	027, 3–4	26th	84	2	17.79	17.31	7.86	16.82
11	028	26th	73	1	17.65	17.00	8.05	16.80
12	030	27th	89	3	17.55	16.95	7.725	16.70
14	036	27th	87	2	17.775	17.365	7.795	17.35
15	038	27th	75	1	17.75	17.125	8.55	16.075
16	042, 1–1	27th	78	1	17.80	17.175	8.30	16.225
20	048	27th	79	1	17.30	16.70	8.35	17.425
21	051	27th	77	1	17.80	17.175	8.30	16.125
23	058, 3–4	27th	92	3	16.35	15.90	7.625	15.095
24	058, 5–5	27th	94	3	17.525	16.975	7.825	16.525
27	068	28th	85	2	18.14	17.71	7.88	16.075
28	071	28th	74	1	15.85	15.375	7.95	15.30
29	073	28th	76	1	18.20	17.575	8.40	15.65
32	080	28th	81	2	18.485	18.065	7.92	15.835
33	083	28th	96	3	17.45	16.85	7.575	16.695
35	089	28th	88	2	18.205	17.755	7.98	15.91
41	117, 2–2	29th	95	3	17.35	16.80	7.85	16.705
44	123, 1–4	30th	90	3	17.675	17.125	7.975	16.17

[a] As measured by variation in means of two riffles from each bag.

Table A-11. Analysis of variance of instrument samples

(Data of Steps 13, 28, and 43)

(Units in per cent)

Source of Variation	SS	df	ms	Estimated Net Variance Component	Estimated Net Standard Deviation	Estimated 0.95 Confidence Limits
Fertilizer A: 10–10–10, Granulated						
			Nitrogen[a]			
Blocks	2.2326	1	2.2326	0.0934	0.31	0–10.43
Days *w.* Blocks	0.0636	2	0.0318	0.0015	0.04	0– 0.35
Bags *w.* Blocks	2.7903	8	0.3488	0.0824	0.29	0.13– 0.61
Samples *w.* Bags	0.1419	10	0.0142	0.0013	0.04	0– 0.14
Bags × Days *w.* Blocks	0.1333	8	0.0167	0.0025	0.05	0– 0.17
Residual	0.1169	10	0.0117	0.0117	0.11	0.08– 0.19
			Total Phosphorus (P_2O_5)			
Blocks	0.2677	2	0.1339	0	0	0– 0.39
Days *w.* Blocks	0.2476	3	0.0825	0.0053	0.07	0.03– 0.28
Bags *w.* Blocks	18.7511	18	1.0417	0.2575	0.51	0.32– 0.82
Samples *w.* Bags	0.2708	21	0.0129	0.0020	0.04	0– 0.10
Bags × Days *w.* Blocks	0.1384	18	0.0077	0	0	0– 0.07
Residual	0.1889	21	0.0090	0.0090	0.09	0.07– 0.13
			Available Phosphorus[b] (P_2O_5)			
Blocks	0.5009	2	0.2505	0	0	0– 0.61
Days *w.* Blocks	0.1585	3	0.0528	0.0038	0.06	0.02– 0.25
Bags *w.* Blocks	15.4323	15	1.0288	0.2540	0.50	0.30– 0.85
Samples *w.* Bags	0.2166	18	0.0120	0.0031	0.06	0– 0.11
Bags × Days *w.* Blocks	0.1030	15	0.0069	0.0005	0.02	0– 0.08
Residual	0.1056	18	0.0059	0.0059	0.08	0.06– 0.11
			Potash			
Blocks	1.4015	2	0.7008	0	0	0– 0.97
Days *w.* Blocks	0.0372	3	0.0124	0	0	0– 0.10
Bags *w.* Blocks	23.4449	18	1.3025	0.3145	0.57	0.35– 0.92
Samples *w.* Bags	0.5448	21	0.0259	0.0058	0.08	0– 0.16
Bags × Days *w.* Blocks	0.5923	18	0.0329	0.0093	0.10	0– 0.19
Residual	0.3009	21	0.0143	0.0143	0.12	0.09– 0.17
Fertilizer B: 0–20–20, Powdered						
			Total Phosphorus (P_2O_5)			
Blocks[c]	0.0686	1	0.0686	0	0	0– 1.51
Days *w.* Blocks	0.0030	2	0.0015	0	0	0– 0.01
Bags *w.* Blocks	40.9005	12	3.4084	0.8251	0.91	0.50– 1.65
Samples *w.* Bags	0.9646	14	0.0689	0.0258	0.16	0.05– 0.31
Bags × Days *w.* Blocks	0.6775	12	0.0565	0.0196	0.14	0.02– 0.29
Residual	0.2435	14	0.0174	0.0174	0.13	0.10– 0.21
			Available Phosphorus (P_2O_5)			
Blocks[c]	1.5545	1	1.5545	0	0	0– 7.36
Days *w.* Blocks	0.0047	2	0.0024	0	0	0– 0.05
Bags *w.* Blocks	35.8593	12	2.9883	0.7217	0.85	0.47– 1.54
Samples *w.* Bags	0.9090	14	0.0649	0.0212	0.15	0– 0.29
Bags × Days *w.* Blocks	0.7118	12	0.0593	0.0184	0.14	0– 0.29
Residual	0.3166	14	0.0226	0.0226	0.15	0.11– 0.24

(Continued)

Table A-11 (*Continued*)

Source of Variation	SS	df	ms	Estimated Net Variance Component	Estimated Net Standard Deviation	Estimated 0.95 Confidence Limits
Fertilizer B: 0–20–20, Powdered						
Potash						
Blocks[d]	—	—	—	—	—	—
Days *w.* Blocks	0.0448	1	0.0448	0	0	0– 1.73
Bags *w.* Blocks	34.0066	6	5.6678	1.4080	1.19	0.51– 2.84
Samples *w.* Bags	0.3893	7	0.0556	0	0	0– 0.32
Bags × Days *w.* Blocks	0.3404	6	0.0567	0	0	0– 0.35
Residual	0.5282	7	0.0755	0.0755	0.28	0.18– 0.56
Fertilizer C: 5–10–10, Powdered						
Nitrogen						
Blocks[e]	0.0825	1	0.0825	0.0016	0.04	0– 1.70
Days *w.* Blocks	0.0537	2	0.0269	0.0017	0.04	0.01– 0.27
Bags *w.* Blocks	0.1783	12	0.0149	0.0019	0.04	0– 0.14
Samples *w.* Bags	0.0744	14	0.0053	0.0018	0.04	0.01– 0.08
Bags × Days *w.* Blocks	0.0432	12	0.0036	0.0010	0.03	0– 0.07
Residual	0.0243	14	0.0017	0.0017	0.04	0.03– 0.07
Total Phosphorus (P_2O_5)						
Blocks[d]	—	—	—	—	—	—
Days *w.* Blocks	0.0804	1	0.0804	0.0030	0.05	0– 2.32
Bags *w.* Blocks	0.6186	6	0.1031	0.0180	0.13	0– 0.37
Samples *w.* Bags	0.0925	7	0.0132	0.0016	0.04	0– 0.17
Bags × Days *w.* Blocks	0.1671	6	0.0279	0.0090	0.09	0– 0.27
Residual	0.0700	7	0.0100	0.0100	0.10	0.07– 0.20
Available Phosphorus (P_2O_5)						
Blocks[d]	—	—	—	—	—	—
Days *w.* Blocks	0.0432	1	0.0432	0.0006	0.02	0– 1.70
Bags *w.* Blocks	0.4431	6	0.0739	0.0081	0.09	0– 0.31
Samples *w.* Bags	0.0962	7	0.0137	0.0030	0.05	0– 0.17
Bags × Days *w.* Blocks	0.2130	6	0.0355	0.0139	0.12	0– 0.31
Residual	0.0538	7	0.0077	0.0077	0.09	0.06– 0.18
Potash						
Blocks[d]	—	—	—	—	—	—
Days *w.* Blocks	0.0055	1	0.0055	0	0	0– 1.92
Bags *w.* Blocks	0.3968	6	0.0661	0.0140	0.12	0– 0.30
Samples *w.* Bags	0.1200	7	0.0171	0.0019	0.04	0– 0.17
Bags × Days *w.* Blocks	0.0396	6	0.0066	0	0	0– 0.11
Residual	0.0938	7	0.0134	0.0134	0.12	0.08– 0.24

[a] Based on the 1st, 2nd, 4th, 5th, and 7th bags tested by Laboratory 3 and on five bags selected at random from the 1st, 2nd, 3rd, 5th, 6th, and 7th bags tested by Laboratory 1. None of the results reported by Laboratory 2 were used because of the excessive heterogeneity in the day factor. *See* Chapter IV, Section 2, "Day-to-Day Variations as Estimated from Two Days a Week Apart."

[b] Based on the last six bags tested by Laboratory 2, and six bags selected at random from the seven bags tested by Laboratories 1 and 3. *See* Chapter IV, Section 2 "Day-to-Day Variations as Estimated from Two Days a Week Apart."

[c] Laboratories 2 and 3 only.

[d] Based on Laboratory 3 only.

[e] Based on Laboratories 1 and 3.

Table A-12. Analysis of variance of riffle samples (Data of Steps 15, 30, and 45)

(Units in per cent)

Source of Variation	SS	df	ms	Estimated Net Variance Component	Estimated Net Standard Deviation	Estimated 0.95 Confidence Limits
Fertilizer A: 10–10–10, Granulated						
			Nitrogen			
Blocks	3.4617	2	1.7309	0.1057	0.33	0.15–2.06
Bags *w.* Blocks	0.8450	21	0.0402	0.0085	0.09	0–0.19
Samples *w.* Bags[a]	0.5600	24	0.0233	0.0233	0.15	0.12–0.21
			Total Phosphorus (P_2O_5)			
Blocks	0.3650	2	0.1825	0	0	0–0.65
Bags *w.* Blocks	8.4275	21	0.4013	0.1990	0.45	0.29–0.69
Samples *w.* Bags[a]	0.0800	24	0.0033	0.0033	0.06	0.05–0.08
			Available Phosphorus (P_2O_5)			
Blocks	0.2554	2	0.1277	0	0	0–0.54
Bags *w.* Blocks	7.0544	21	0.3350	0.1660	0.41	0.26–0.63
Samples *w.* Bags[a]	0.0950	24	0.0040	0.0040	0.06	0.05–0.09
			Potash			
Blocks	0.3004	2	0.1502	0	0	0–0.09
Bags *w.* Blocks	9.4644	21	0.4507	0.2099	0.46	0.29–0.72
Samples *w.* Bags[a]	0.7450	24	0.0310	0.0310	0.18	0.14–0.25
Fertilizer B: 0–20–20, Powdered						
			Total Phosphorus (P_2O_5)			
Blocks	2.1530	2	1.0765	0.0060	0.08	0–1.61
Bags *w.* Blocks	20.6063	21	0.9813	0.4082	0.64	0.36–1.04
Samples *w.* Bags[a]	3.9600	24	0.1650	0.1650	0.41	0.32–0.57
			Available Phosphorus (P_2O_5)			
Blocks	1.0888	2	0.5444	0	0	0–1.13
Bags *w.* Blocks	19.9137	21	0.9483	0.3965	0.63	0.36–1.03
Samples *w.* Bags[a]	3.7300	24	0.1554	0.1554	0.39	0.31–0.55
			Potash			
Blocks	5.4087	2	2.7044	0.0695	0.26	0–2.55
Bags *w.* Blocks	33.4544	21	1.5931	0.6811	0.83	0.48–1.33
Samples *w.* Bags[a]	5.5450	24	0.2310	0.2310	0.48	0.38–0.67
Fertilizer C: 5–10–10, Powdered						
			Nitrogen			
Blocks	0.4731	2	0.2369	0.0100	0.10	0–0.76
Bags *w.* Blocks	1.5994	21	0.0762	0.0334	0.18	0.11–0.29
Samples *w.* Bags[a]	0.2250	24	0.0094	0.0094	0.10	0.08–0.13
			Total Phosphorus (P_2O_5)			
Blocks	0.2150	2	0.1075	0	0	0–0.51
Bags *w.* Blocks	2.2832	21	0.1087	0.0374	0.19	0.08–0.34
Samples *w.* Bags[a]	0.8150	24	0.0340	0.0340	0.18	0.14–0.26

(Continued)

Table A-12 (*Continued*)

Source of Variation	SS	df	ms	Estimated Net Variance Component	Estimated Net Standard Deviation	Estimated 0.95 Confidence Limits
Fertilizer C: 5–10–10, Powdered						
			Available Phosphorus (P_2O_5)			
Blocks	0.4513	2	0.2257	0.0080	0.09	0–0.55
Bags *w.* Blocks	2.0319	21	0.0970	0.0311	0.18	0.07–0.26
Samples *w.* Bags[a]	0.8350	24	0.0348	0.0348	0.19	0.18–0.33
			Potash			
Blocks	0.0237	2	0.0119	0	0	0–0.53
Bags *w.* Blocks	2.0044	21	0.0954	0.0357	0.19	0.09–0.32
Samples *w.* Bags[a]	0.5750	24	0.0240	0.0240	0.16	0.12–0.22
Fertilizer D: 10–10–10, Granulated						
			Nitrogen			
Blocks	2.2829	2	1.1415	0.0682	0.26	0.16–1.67
Bags *w.* Blocks	1.0669	21	0.0508	0.0166	0.13	0.05–0.23
Samples *w.* Bags[a]	0.4250	24	0.0177	0.0177	0.13	0.10–0.19
			Total Phosphorus (P_2O_5)			
Blocks	2.3579	2	1.1790	0.0391	0.20	0–1.69
Bags *w.* Blocks	11.6319	21	0.5539	0.2642	0.51	0.33–0.80
Samples *w.* Bags[a]	0.6150	24	0.0256	0.0256	0.16	0.12–0.22
			Available Phosphorus (P_2O_5)			
Blocks	3.7305	2	1.8653	0.0859	0.29	0–2.14
Bags *w.* Blocks	10.3187	21	0.4914	0.2343	0.48	0.31–0.76
Samples *w.* Bags[a]	0.5500	24	0.0229	0.0229	0.15	0.12–0.21
			Potash			
Blocks	3.0013	2	1.5007	0.0300	0.17	0–1.90
Bags *w.* Blocks	21.4213	21	1.0201	0.2913	0.54	0.03–1.00
Samples *w.* Bags[a]	1.0500	24	0.0438	0.0438	0.21	0.16–0.29

[a] Actually a combination of within-bag variation and within-day laboratory test error.

Table A-13. Steps in the computation of the net components of variance of Table VI-4

Chemical Elements	N	TP	AP	K
1. *Computation of Variance of Mean of 20 bags*				
(a) Net bag-to-bag variance (from Table A-11)				
A	0.0824	0.2575	0.2540	0.3145
B	—	0.8251	0.7217	1.4080
C	0.0017	0.0180	0.0081	0.0140
(b) Net instrument sampling variance (from Table A-11)				
A	0.0013	0.0020	0.0031	0.0058
B	—	0.0258	0.0212	0
C	0.0018	0.0016	0.0030	0.0019
(c) Sum of (a) and (b)				
A	0.0837	0.2595	0.2571	0.3203
B	—	0.8509	0.7429	1.4080
C	0.0035	0.0196	0.0111	0.0159
(d) (c) divided by 20				
A	0.0042	0.0130	0.0129	0.0160
B	—	0.0425	0.0371	0.0704
C	0.0002	0.0010	0.0006	0.0008
2. *Computation of Net Riffling Variance*				
(a) Mean of 21 ranges of samples of two (Tables III-8 to III-11)				
A	0.125	0.057	0.070	0.184
B	—	0.362	0.373	0.414
C	0.091	0.197	0.187	0.144
(b) (a) divided by 1.14 (*See* p. 117 and Table E2 of A. J. Duncan, *Quality Control and Industrial Statistics, 2nd Ed.*)				
A	0.110	0.050	0.061	0.161
B	—	0.318	0.327	0.363
C	0.080	0.173	0.164	0.126
(c) (b^2)				
A	0.0121	0.0025	0.0037	0.0259
B	—	0.1011	0.1069	0.1318
C	0.0064	0.0299	0.0269	0.0159
(d) Variance for tests run the same day (Table IV-3 and Table A-11)				
A	0.0100	0.0090[a]	0.0055	0.0039
B	—	0.0174[a]	0.0130	0.0155
C	0.0017[a]	0.0100[a]	0.0077[a]	0.0134[a]
(e) (c) minus (d). (Taken as 0 if (d) > (c). Result = Net Variance of Riffling)				
A	0.0021	0	0	0.0220
B	—	0.0837	0.0939	0.1163
C	0.0047	0.0199	0.0192	0.0025
3. *Computation of Variance of Mean of Two Determinations Run on Different Days*				
(a) Net day-to-day variance[b] (Table IV-3 and Table A-11)				
A	0.0133	0.0053[a]	0.0042	0.0044
B	—	0.0629[c]	0.0629	0.0073
C	0.0017[a]	0.0030[a]	0.0006[a]	0[a]

(Continued)

Table A-13 (*Continued*)

Chemical Elements	N	TP	TP	K
(b) Sum of (a) of Section 3 and (d) of Section 2				
A	0.0233	0.0143	0.0097	0.0083
B	—	0.0803	0.0759	0.0228
C	0.0034	0.0130	0.0083	0.0134
(c) (b) divided by 2 = Variance of mean of sample of two				
A	0.0117	0.0072	0.0049	0.0042
B	0.0000	0.0402	0.0380	0.0114
C	0.0017	0.0065	0.0042	0.0067
4. *Computation of Net Interlaboratory Variance* (Based on Table A-9 with laboratories treated as if they had been selected at random from the population of state laboratories. No allowance made for finiteness of the population.)				
(a) Lab. m.s.				
A	59.72	19.06	30.50	0.39
B	—	20.67	52.72	2.06
C	8.72	15.17	23.17	12.39
(b) Lab × bag m.s.				
A	6.12	4.55	2.17	4.11
B	—	4.67	9.31	5.06
C	0.89	1.34	5.34	19.89
(c) (a) minus (b)				
A	53.60	14.51	28.33	0[d]
B	—	16.00	43.41	0[d]
C	7.78	13.83	17.83	0[d]
(d) (c) divided by 600 (Each lab. made 6 tests. The factor 100 is needed to decode the data to units of one per cent.) = Net interlaboratory variance				
A	0.0893	0.0242	0.0472	0
B	—	0.0267	0.0724	0
C	0.0131	0.0231	0.0297	0

[a] Table A-11.
[b] To compute the net standard deviation from Table IV-3, subtract the test error mean square from the Bet. Days *w.* Labs. mean square and divide by 2.
[c] Taken the same as the variance for AP.
[d] Any negative results taken as zero.

BOOK REVIEWS

United States Pharmacopeia, Sixteenth Revision (USP XVI), Official October 1, 1960. United States Pharmacopeial Convention, Inc. Distributor: Mack Publishing Company, Easton, Pa. xliv + 1148 pages. Price $10.00 domestic, $10.50 foreign.

The United States Pharmacopeia is a 140-year-old institution. The changes in succeeding revisions are of an evolutionary nature —very slow, but definitely in a forward direction. The original USP was published in Latin and English. Latin titles were retained as the principal headings of monographs until the 13th Revision, in 1947, when they were relegated to secondary status; they remained there until they were finally deleted in the 15th Revision (1955). Other than this, the last significant change in format was the inclusion of structural formulae, in the 11th Revision (1936).

Aside from format, however, an examination of the content of succeeding revisions will show that the Pharmacopeia is dynamic, keeping pace with the precipitous rate of change in modern therapeutics, adopting the important new drugs and dropping others as their importance decreases. The USP XVI contains 908 individual monographs, compared with 838 in USP XV. These include 225 new admissions, with 159 deletions from USP XV. Of these 159 deletions, 43 had been newly admitted to USP XIV (1950), 42 to USP XV (1955) and one as recently as the 1st Supplement to USP XV.

In addition to the new admissions there are 81 interim admissions of articles which have been approved for admission by the Subcommittee on Scope, but for which monographs were not prepared because suitable assays, tests, and standards had not been developed in time for publication.

The trend toward inclusion of patented preparations is continuing. In USP XIV, in which the practice of designating these was inaugurated, 49 monographs of patented items were included. The number increased to 75 in USP XV, and to a total of 90 in USP XVI.

In view of the extensive review to which the manuscripts, galleys, and proofs are subjected to prior to publication, it is difficult to detect errors in the text. One non-typographical error is found in the structural formula of histamine phosphate; the feebly basic imino-nitrogen, rather than the more strongly basic amino nitrogen in the imidazole ring, is depicted in the cation center.

Two very welcome changes appear in the appropriate monographs. Many of the assays which previously consisted simply of spectrophotometric measurement of a solution of the sample of the dosage form, with no preliminary purification other than filtration, have been revised to require an isolation and purification step. More important from both theoretical and practical standpoints is the requirement that the calculations be based upon comparison of the absorbance of the sample solution with that of a solution of a USP Reference Standard, rather than with a given absorbance value.

A continuation of this type of revision is anticipated.

It is essential that modernization of the USP continue. Several of the analytical methods for older drugs should be revised, and the cumbersome antiquated procedures replaced by modern techniques.

JOSEPH LEVINE

Organic Reactions, Volume 10. Roger Adams, Editor-in-Chief. John Wiley & Sons, Inc., New York, N.Y., 1959. vii + 563 pp. $12.00.

The tenth volume of this well known series maintains the high standard the organic chemist has come to expect in these classics over a period of nearly two decades. The current volume deals with the mechanism, scope, limitations, synthetic applications, experimental conditions, and procedures for

three important reactions and includes the usual thoroughly documented tabular surveys of each.

The first chapter of 142 pages by Stanley M. Parmerter covers the coupling of diazonium salts with aliphatic carbon atoms and provides 480 references, including reactions recorded up to about 1956, with several more recent examples.

Quite appropriately Chapter 2 deals with the Japp-Klingemann reaction, the diazonium salt coupling with an activated methinyl carbon which results in splitting out of a group to produce a hydrazone instead of the azo compound usually obtained with aliphatic carbons. This treatise of 36 pages by Robert R. Phillips includes 118 references to this special case—again up to 1956.

The remainder of the volume is devoted to a comprehensive survey of the Michael condensation, one of the most important organic reactions. This chapter by Ernst D. Bergmann, David Ginsburg, and Raphael Pappo presents 1045 references, including a number as late as 1958. The monumental tabular survey of 273 pages is very well organized. The basic classification involves the unsaturated acceptor molecules listed in order of increasing number of carbon atoms. The reaction of each acceptor with a systematized series of donors then greatly facilitates the location of a specific reaction in the extensive tables. A separate table of the important donors extends the usefulness of the survey.

No further commendation is necessary than to emphasize that the current volume is most worthy to join its invaluable, time-saving predecessors in this series which is expected universally to be available in any chemical library worthy of the name.

EDWARD O. HAENNI

Radiometric Trace Analysis of Lead. By P. C. VanErkelens. Drukkery H. J. Smits, Utrecht, Netherlands, 1960.

This paperback volume of 104 pages is an English translation of an academic dissertation for the Doctorate degree. The author introduces his subject with seven pages of discussion of nuclear energy uses in analysis. Similar information may be found in radiochemical textbooks. However, the author has provided a unique tabulation "Comparison of Analytical Methods Using Nuclear Energy as a Tool" that rearranges and collects well-known data; and condenses, collates, and presents it concisely and succinctly. There is an abridged literature survey of radioreactions and radioexchange analyses.

The purpose of this work was to test the applicability of some radiochemical techniques and to develop methods for determination of microgram quantities (.05–10 mmg) of lead in biological materials. The methods that were developed did not fully realize this goal since the largest samples were of the order of 50 mg which were destroyed by dry-ashing or wet-ashing (HNO_3, H_2O_2) or both. Thus the lowest level of lead determined is 1 ppm. The lead content of most biological materials is below this level therefore other methods of analysis would generally be selected.

The volume is useful for chemists that may be attempting development of radioreaction and radiochemical methods. It describes the methods that failed and reasons therefore as well as the successes. Many techniques related to the main project were tested and applied. For example:

(1) Preparation of labeled organic reagents H_2S^{35}, mercaptobenzthiazole-S^{35}. Acetylacetone-C^{14}, diethyldithiocarbamate-S^{35}

(2) Separation of Pb^{210} (RaD) decay products by ion-exchange on Dowex 1 × 10 in HCl.

(3) Chromatographic separations of metals on "Al_2O_3-glass paper"

Two chapters (III, IV) are devoted to details of development of a chromatographic method for separation of lead (in ash) from interfering cations, conversion to $PbHP^{32}O_4$ and subsequent measurement of the radioactivity in the region R_F 0.1–0.2. The method measures 1 mmg of Pb in the presence of 100-300 mmg mixed cations Ca^{++}, Cu^{++}, Zn^{++}, and anions PO_4^{---}, SO_4^{--} with a standard deviation of ±10%.

Chapter III describes the work on exchange of Pb^{++} with Tl^{204}-diethyldithiocarbamate complex in CCl_4. This method was

the most successful of all the attempts. However only 80–90% of amounts of lead (less than 3 mmg) were exchanged during a reasonable shaking period (6 minutes) and with reasonable excess (2½ to 20 fold) of reagents. Isotope dilution technique with Pb^{210} was introduced to measure the total exchange. Since two isotopes had to be measured simultaneously the Tl^{204} was counted with a Geiger Mueller tube for liquid samples. Pb^{210} was counted with a well-type sodium iodide crystal.

Unfortunately in this method there is very little discussion of interfering substances. The impression is given that the method is highly selective. The procedure presumably has been tested with samples containing 0.1–0.6 mmg of lead "with less than 0.1 fold (gram-equivalent) quantities of bismuth and thallium, a 6.0, 8.0, 28.0 fold amount of Fe, Cu (or Ag or Hg), and Zn (or Mn, etc.) respectively; and less than 15 fold PO_4^{---} (or $P_2O_7^{---}$).

The method is rapid (40 minutes, not including calculations) in the range of .05 to 0.6 mmg with standard deviation 12% at 0.5 mmg and 7–9% at higher levels.

HOWARD M. BOLLINGER

Flame Photometry. By John A. Dean. McGraw-Hill Book Company, Inc., New York, N.Y., 1960. vi + 354 pp. Illus., index, bibl. $11.50.

This book is the first full-length treatment (a comprehensive treatise) of the principles and practical applications of modern flame photometry. Its language is clear and concise.

This treatise provides a mass of fundamental material for research and control chemists interested in this phase of instrumentation. The scope is such that it covers in great detail the essential principles of flame photometry and the behavior of approximately 35 elements that can be excited to emit detectable radiation in a flame. Included are many graphs, spectrograms, and tables to help illustrate the text. Also, this work contains ample material for a college course in flame photometry; and laboratories employing this type of instrumentation will find the book an extremely useful reference. The author's treatment of the individual elements is outstanding. The subject matter, based on the latest electronic techniques, is presented in a way readily understood by the average chemist.

The book is organized in four sections. Part 1 deals with the principles of atomization, flame characteristics, the excitation of metallic spectra, and the use of organic solvents. Part 2 discusses the flame photometer, interferences, evaluation methods, selection of optimum working conditions, and absorption flame photometry. Part 3 presents an introduction to the determination of the elements, the alkali metals, the alkaline earth metals, elements of the periodic groups I–B, II–B, and IV, the platinum metals, the rare earths, scandium, yttrium, lanthanum, boron, aluminum, indium, gallium, thallium, chromium, molybdenum, vanadium, manganese, iron, cobalt, nickel, and the nonmetals. Part 4 includes clinical applications, agronomic analyses, and the analyses of glass and cement. The appendix of this book includes directions for preparing stock solutions and tabulates analytical factors most commonly used in this type of work. The author lists 780 references with title, author, and journal.

With chemical research and control on the increase, the need for flame photometric procedures to help reduce costs and time will continue to rise. Therefore, it is the reviewer's opinion that the information included in this book will contribute materially toward these objectives.

MAYNARD J. PRO

X-Ray Absorption and Emission in Analytical Chemistry. By Liebhafsky, H. A., Pfeiffer, H. G., Winslow, E. H., and Zemany, P. D. John Wiley and Sons, Inc., 1960. $13.50.

In the preface the authors state, "This book was written for the analytical chemist who wants to use these X-ray methods and to understand them. We have striven for correctness in physics, electronics and statistics; but we have tried first of all to help

the analytical chemist in his work." The reviewer has often seen similar expressions of hope in books on instrumental analysis; far too frequently such books fail to achieve the stated objective.

These authors have written a book which is of considerable value to the analytical chemist who wants to *use* and to *understand* X-ray methods. The book should be quite valuable to those who are just starting in this rapidly expanding field and to those who wish to evaluate the possible application of X-ray methods to their particular problems.

The book is not easy reading for chemists without a sound basic training in physics, electronics, and statistics. On the whole, however, the authors' explanations of these basic topics are sound, clear, and concise. Careful study of the authors' explanations will be quite helpful to the chemist.

The major limitations of the book from the chemist's standpoint are that few references to specific methods are given and that most of the references quoted are for 1957 or earlier. As noted by the authors (page 41), this fault is minimized by the ready availability of the review articles on this subject by one of the authors which appear regularly in *Analytical Chemistry*.

JOHN H. JONES

Soil Fertility and Animal Health. By William A. Albrecht. The Fred Hahne Printing Co., Webster City, Iowa, 1958, 232 pp. $3.98.

"Soil Fertility and Animal Health" was first published as a series of articles in the *Aberdeen-Angus Journal*. The book summarizes much of the work of the author and associates during the past three decades. A great deal of useful information with respect to soil chemistry, plant composition, and animal health is given.

The book emphasizes the influence of precipitation on soil fertility and character of native vegetation. Adequate supplies of calcium and phosphorus in vegetation have an important effect on other features of plant composition. Magnesium has recently come in for a place of importance in the soil fertility-plant composition picture.

Many readers will agree with most of the statements of the author, but some will want more proof before accepting the theory that "only fertile soils grow complete proteins as natural guard against diseases."

Anyone interested in the field of animal nutrition should find this an interesting volume.

M. S. ANDERSON

Taxonomy of Flowering Plants. By C. L. Porter. W. H. Freeman and Co., San Francisco, Calif.; 1959. xii + 452 pp. 600 Illus. $6.95.

This text was written for those students who wish to acquire some general knowledge of plant classification. It saves them going through the usual detailed discussions on this topic which is so thoroughly covered in the excellent textbooks on this subject.

The material is presented in this text by the author in a systematic and comprehensible terminology. The book is well illustrated with floral diagrams and excellent photographs illustrating the various types of flowering plants representative of the North American flora.

The text is divided into three parts viz., I: Historical and Morphological Aspects of Taxonomy; II: Selected Orders and Families of Monocotyledons; III: Selected Orders and Families of Dicotyledons.

Part I. This portion, which contains 9 chapters, discusses the historical background of taxonomy, the literature in the field of taxonomy, the subject of collecting and preserving specimens, the explanation and definition of nomenclature, and the use and development of keys. It contains drawings and diagrams of the various parts of the flower pattern and is excellent background for the material presented in parts II and III. A list of references is given at the end of each chapter for those whose interest has been stimulated to investigate these topics more fully.

Parts II and III contain only descriptive material of selected orders and families. Those of the monocotyledons are in part II and those of the dicotyledons in Part III.

Only certain orders and families of each of these two large groups are described. Examples of each order and family described are presented, and floral diagrams along with photographs of many of the examples cited illustrate the data. In part II the author has described some 19 orders and 23 families of the monocotyledons and some 35 orders and 80 families of the dicotyledons in part III.

The author concludes the book with a 16-page glossary explaining botanical terms used in the text.

This is an excellent text for its intended use as an opening "wedge" to this broad topic of plant taxonomy.

F. ALLEN HODGES

ANNOUNCEMENTS

Alcoholic Beverages:

Raymond Nelson, Alcohol and Tobacco Tax Division, Internal Revenue Service, Washington 25, D.C., has been appointed Associate Referee on Phosphates in Wines.

A. P. Mathers, Alcohol and Tobacco Tax Division, Internal Revenue Service, Washington 25, D.C., has resigned as Associate Referee on Methanol (Chemical).

Drugs in Feeds:

Anthony Abbey, Product Laboratory, American Cyanamid Co., Princeton, N. J., has been appointed Associate Referee on Chlortetracycline.

George E. Keppel, Food and Drug Administration, Cincinnati 2, Ohio, has resigned as Associate Referee on Schradan.

Metals, Other Elements, and Residues in Foods:

George McClellan, Fishery Products Technologist, U.S. Department of the Interior, Fish and Wildlife Service, P.O. Box 630, Pascagoula, Mississippi, has been appointed Associate Referee on Tin in Foods.

Miscellaneous Drugs:

Mason P. Goldman, Food and Drug Administration, Post Office Building, Room 415, Buffalo 3, N.Y., has been appointed Associate Referee on Separation of Mixtures of Hormones.

Pesticides:

David Leeper, Organic Materials Co., P.O. Box 1150, McAllen, Texas, has been appointed Associate Referee on Pentachlorophenol.

John T. Ford, Analytical Division, Research Center, Hercules Powder Co., Wilmington, Delaware, has been appointed Associate Referee on Toxaphene.

Richard P. Gigger, Geigy Research Laboratories, P.O. Box 430, Yonkers, N.Y., has been appointed Associate Referee on DDT.

Vegetable Drugs and Their Derivatives:

Joseph Levine, Food and Drug Administration, Washington 25, D.C., has been appointed General Referee of this division.

William T. Butler, Alcohol and Tobacco Tax Division, Internal Revenue Service, Washington 25, D.C., has been appointed Associate Referee on Marijuana.

CORRECTIONS

This Journal, **43,** 409 (1960), Add "Sample Powder I," "Sample Powder J," and "Sample Powder K" as subheads, to lower portion of Columns, 1, 2, and 3 respectively, Table 3.

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