First Day

MONDAY—MORNING SESSION

REPORT ON PROCESSED VEGETABLE PRODUCTS

By L. M. Beacham (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Referee*

During the year work was continued on a rapid method for measuring catalase activity, and the results look promising. Samples of frozen peas and frozen asparagus were prepared and sent out to collaborating analysts. However, since this could not be done until the season for these vegetables came around in June, the samples did not reach the collaborators in time for all of the results to be reported for the 1952 meeting. The Referee recommends,* therefore, that the rapid method be kept under study for another year.

Study of acetaldehyde as an index of quality in frozen vegetables has continued actively since the last meeting. The studies have been focused on perfecting a method for detecting and measuring a few parts per million of acetaldehyde in frozen peas and asparagus, and the Associate Referee has under study a method that gives highly satisfactory results in his hands and in those of his colleagues in the same laboratory. Difficulty has been encountered in preparing uniform samples of frozen vegetables for collaborative studies. This year it was decided to submit to the collaborators pure solutions of acetaldehyde at various concentration levels and have the collaborators mix them into samples of frozen vegetables purchased locally. The vegetables were first tested for naturally occurring acetaldehyde and again tested after addition of specified amounts of the solutions. Results were erratic, and further investigation showed that the dilute acetaldehyde solutions had been subjected to attack by molds. The referee recommends* that the studies of this subject be continued for another year.

The referee likewise concurs in the recommendation* of the Associate Referee on peroxidase in frozen vegetables, that the ascorbic acid oxidation method for measuring peroxidase be subjected to further study during the coming year.

REPORT ON PEROXIDASE IN FROZEN VEGETABLES

By M. A. Joslyn (Department of Food Technology, University of California, Berkeley 4, Calif.), *Associate Referee*

Methods of enzyme assay, particularly those proposed for ferruginous or iron enzymes (e.g., catalase and peroxidase) rarely have been subjected to collaborative study. There is some evidence in the literature that a method developed for one particular agricultural product may not be applicable to others. Balls (1) reported that the results obtained for the

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 54 (1953).

catalase content of two samples of flour sent to seven collaborators together with instructions based upon the method for catalase in leaves (2) were not satisfactory. While individual operators duplicated their own work the results obtained from various laboratories differed too greatly, apparently due to some manipulative detail. Subsequently, Lineweaver and Morris (3) modified this method for catalase particularly to avoid obscurance of the end point in the thiosulfate titration of the iodine liberated from potassium iodide by the residual hydrogen peroxide, and subjected it to collaborative trial with frozen vegetables. Results on well-blanched string beans, peas, and on the mildly blanched vegetables in which only 70–97 per cent of the catalase was destroyed, were satisfactory. This method is included as a method for catalase in vegetables in the 7th edition of *Methods of Analysis* (4).

The general problems involved in preparation of extracts and peroxidase determination were discussed in our first report (5). The possible multiple nature of the naturally occurring vegetable peroxidases was pointed out. This was confirmed recently by Jermyn (6) who found four components in purified horseradish peroxidase. Keilin and Hartree (7) reported additional data on the chemical structure of horseradish peroxidase. They reported that in the purpurogallin test method, no commercially available samples of pyrogallol were satisfactory unless resublimed under reduced pressure. George (8) recently reported that horse-radish peroxidase forms three distinct intermediates with hydrogen peroxide. Data on the chemistry of other plant peroxidases is still unavailable.

The possibility of interference by catalase in the determination of peroxidase activity in vegetable extracts has been investigated only in the titrimetric pyrogallol procedures. Balls and Hale (9) reported that at the slightly alkaline *p*H (*p*H 8) used in their titrimetric procedure for the determination of peroxidase in agricultural products there was no interference from catalase even when considerable amounts were present. Morris, *et al.* (10) concluded that carrot catalase did not interfere in the determination of carrot peroxidase by the titrimetric guaiacol procedure. The liberation of oxygen by carrot catalase was inhibited more than 95 per cent by guaiacol. The peroxidase activity of carrot extracts, determined by the decrease in peroxide, agreed with the activity determined colorimetrically by the increase in guaiacol oxidation product. Neither Balls and Hale (9) nor Morris, *et al.* (10), however, actually determined the effect of addition of catalase to their vegetable extracts as Balls and Hale (2) did, for example, when they proved that the addition of horseradish peroxidase had no effect on catalase determination. Whether guaiacol would inhibit catalase activity in extracts of vegetables other than carrots under the conditions of the titrimetric guaiacol peroxidase determinations is not known. Morris, *et al.* (10) do report that horse-radish

catalase was only 50 per cent inhibited by guaiacol and that in a mixture of potato peroxidase and liver catalase the rate of disappearance of peroxidase from the reaction mixture was greater than the rate of appearance of color.

The results of the first collaborative trial of the peroxidase assay in frozen green asparagus, green peas, green pea hulls, and spinach, raw and scalded for 2 minutes at 180°F. and 212°F., were not satisfactory (11). The residual peroxidase activity in the samples was too low, and the scope of the work planned was too extensive for the time available. The analyses, however, were continued subsequently in our laboratory (by Mr. P. Townsley) and the data obtained, which were not reported in 1951, are presented here. In addition, more detailed collaborative trials made on lima beans at our laboratory and that of the Western Regional Research Laboratory in 1952 are presented.

COMPARISON OF METHODS OF EXTRACTION AND PROCEDURES PREVIOUSLY DESCRIBED*

Comparison of Colorimetric Guaiacol Procedures. The three colorimetric guaiacol procedures previously described (methods 1a, 1b, and 1c, reference 11) were compared using 2 per cent NaCl-$CaCO_3$ extractions (Extraction Method 1). The procedures differed in *p*H and in concentration of H_2O_2 and of guaiacol. Klett-Summerson colorimeter readings were taken over a period of 10 minutes. When plotted as colorimeter readings against time the data exhibited certain general patterns: (1) some material containing relatively little residual peroxidase activity (such as pea pods blanched at 212°F. and asparagus blanched at 180°F.) and some containing much (unblanched spinach, unblanched asparagus) gave plots which were linear with time over a period ranging from 400 seconds for raw spinach and 600 seconds for raw asparagus, to over 700 seconds for pea pods blanched at 212°F.; (2) in most cases the initial portion of the plot was linear and then the rate of color formation dropped off and the resulting curve flattened out (for unblanched pea pods and peas, only in the first 80 seconds was the plot linear); (3) in some cases there was an initial lag (during the first 20–40 seconds for pea pods blanched at 180°F. and for unblanched peas, during the first 40–80 seconds for asparagus blanched at 180°F. and during 40–340 seconds for peas blanched at 180°F.). The lag varied with the volume of extract used, the enzyme activity of the extract, and the method of analysis. Method 1c resulted in the greatest lag with asparagus blanched at 180°F., but with peas blanched at 180°F., the greatest lag occurred with method 1a, and least with method 1b. Generally method 1b gave greatest activity; 1c gave nearly as much and in some cases greater activity. Method 1a could not be used except for vegetables of high peroxidase activity.

* Data presented here were obtained in our laboratory by P. Townsley, Sept. 10, 1949 to Jan. 31, 1950.

These results are not surprising, for in the combination of method 1a with extraction procedure 1, the peroxidase catalyzed reaction occurred at about *p*H 7 instead of at the optimum *p*H for guaiacol oxidation. As pointed out previously (5) this was taken as 4.6 by Masure and Campbell (12), 5.0 by Ponting and Joslyn (13) and 5.6 by Morris *et al.* (10).

Comparison of Methods of Extraction as Measured by the Colorimetric Guaiacol Procedures. A comparison was made of the effectiveness of six methods of extracting vegetable peroxidases as measured by the results obtained by the three colorimetric guaiacol procedures. The results obtained can be summarized as follows:

Extraction procedure 1 (2% $NaCl+CaCO_3$), without question, was the best method of extraction with all three colorimetric procedures. This method of extraction yielded an extract which was higher in enzyme activity than any other. It has the added advantage in that the extraction solution is simple to prepare and does not involve expensive chemicals.

In the three colorimetric guaiacol procedures, methods of extraction 2 and 3 gave higher activity measurements than method 4 when determined by procedure 1a and 1c. The situation was reversed when the enzyme activity was determined by procedure 1b; here extraction method 4 ranks as high as method 1 in the extraction of peroxidase. Extraction methods 5 and 6 were low in their ability to extract peroxidase. This result is similar to that obtained by Morris, *et al.* (10) for the peroxidase activity of carrots in which they found that aqueous extracts exhibited only two-thirds of the total peroxidase activity of salt extractions.

Effect of Filtration, Standing, and Shaking on Enzyme Activity. The effect of these manipulative details was determined using various extraction methods and determining the peroxidase activity by the colorimetric guaiacol procedure (b).

For 5 ml extracts of green peas blanched at 180°, by extraction method 1 (2% $NaCl+CaCO_3$), the peroxidase activity increased on standing and also on repeated filtration. The extracts were stored at 0°C., and filtered cold through milk pad filters. The peroxidase activity was also found to increase when the extract was allowed to stand for a length of time and then shaken. As shown in Table 1, allowing the extract to stand for 180 seconds and then shaking for 60 seconds produced an activity equal to that obtained by 150 continuous filtrations (the filtrations took approximately 270 seconds).

It is obvious, therefore, that the treatment of the extract is very important if quantitative results are to be obtained. *The length of time between the preparation of the extract and the determination of its activity is important.* Unless such conditions are observed no two analysts will agree.

When this test was repeated using citrate buffer as extractive (number 2 extraction method) it was found that standing did not increase enzyme activity but that filtration and shaking did increase the activity.

TABLE 1.—*Effect of treatment of enzyme extract on colorimeter readings of peas blanched at 180°F.*

Extraction Method 1, Guaiacol Method (b)

TREATMENT	COLORIMETER READING (SECONDS AFTER MIXING)				
	50	100	200	300	400
1 Filtration	20	88	204	288	360
2 Successive filtrations	28	98	217	304	377
6, 12 & 18 Successive filtrations	35	100	226	320	392
50 Successive filtrations	35	118	252	346	418
75 Successive filtrations	35	122	260	350	425
150 Successive filtrations	40	122	264	368	448
1 Filtration, 180 sec standing and 60 sec shaking	40	122	264	364	446
1 Filtration, 270 sec standing	45	122	285	388	468

Inactivation of the enzyme by filtration was found to occur between 75 and 150 filtrations; the solutions obtained after 150 filtrations were less active than after 75 filtrations. A greater length of standing also decreases the enzyme activity when using this method of extraction but the effect was not as great as that observed above.

With extraction method number 4 (phosphate buffer at *p*H 8), shaking increased activity above either the effect of filtration or of standing. Inactivation of the extract by filtration was observed after 25, 50, and 150 filtrations. Standing increased activity above the initial activity of the extract.

Titrimetric Peroxidase Procedures. The data obtained by the modified Balls and Hale method 2a and guaiacol 2b procedures are shown in Table 2. These involved considerable equipment, preparation, technique, and time. The effect of saturating the solution with nitrogen gas and carrying titrations out under a blanket of nitrogen was inconsistent and did not aid in the determination of peroxidase in the vegetable extracts examined. Of the two procedures, the guaiacol procedure was preferred. The results from this procedure were good and could be duplicated fairly well.

When the efficiency of methods of extraction (for pea pods blanched at 212°F.) is compared by the guaiacol procedure, Table 3 shows that procedure number 4 (phosphate buffer) is best, followed by procedure 1 (2% $NaCl+CaCO_3$) and then by procedures 6, 5, 3, and 2 in that order. When the effect of method of extraction was determined by the modified Balls and Hale pyrogallol procedure, extraction method number 3 gave the highest activity, followed in decreasing activity by extraction procedures 4, 2, 1, 6, and 5, respectively. Methods 4 and 2 gave the same activity.

TABLE 2.—*Titrimetric peroxide procedure*

SAMPLE	BLANCH (°F)	EXTRACTION PROCEDURE	ALIQUOT (ML)	AV. P.E. VALUE/MIN./GRAM TISSUE
	(a) Modified Balls and Hale Pyrogallol Procedure			
Asparagus	180	1	5	0.012
	180	1—N_2 gas	5.0	−0.018
	180	1	11.0	0.016
	180	1—N_2 gas	11.0	0.013
	212	1	10.0	0.0025
	212	1—N_2 gas	10.0	0.000
	R.T.	1	2.0	0.477
	R.T.	1—N_2 gas	2.0	0.378
Spinach	R.T.	1	1	0.147
	R.T.	1	2.0	0.195
	R.T.	1—N_2 gas	2.0	0.178
	180	1	10.0	0.0035
	180	1—N_2 gas	10.0	0.023
	212	1	10.0	0.000
	212	1—N_2 gas	10.0	0.000
Peas	R.T.	1	2	1.201
	R.T.	1	0.5	1.935
	R.T.	1—N_2 gas	1.0	1.600
	R.T.	1—N_2 gas	0.5	1.680
	180	1	10.0	0.0278
	180	1—N_2 gas	10.0	0.0408
	212	1	10.0	0.009
	212	1—N_2 gas	10.0	0.010
Pea pods	R.T.	1	0.04	0.687
	R.T.	1	1.0	0.873
	R.T.	1—N_2 gas	1.0	0.658
	180	1	0.3	?
	180	1	1.0	0.187
	180	1	5.0	0.306
	180	1—N_2 gas	2.0	0.219
	212	1	5.0	0.036
	212	1—N_2 gas	5.0	0.037
		(b) Guaiacol Procedure		
Asparagus	R.T.	1	1	0.538
	R.T.	1—N_2 gas	1.0	0.305
	180	1	11.0	0.026
	180	1—N_2 gas	11.0	0.021
	212	1	10.0	0.005
	212	1—N_2 gas	10.0	0.023
Spinach	R.T.	1	2	0.503
	R.T.	1—N_2 gas	2.0	0.385
	180	1	10.0	0.000
	180	1—N_2 gas	10.0	0.045
	212	1	10.0	0.012
	212	1—N_2 gas	10.0	0.007
Peas	R.T.	1	0.5	1.423
	R.T.	1—N_2 gas	0.5	1.513
	180	1	10.0	0.043
	180	1—N_2 gas	10.0	0.049
	212	1	10.0	0.011
	212	1—N_2 gas	10.0	0.000
Pea pods	R.T.	1	0.5	1.816
	R.T.	1—N_2 gas	0.4	1.880
	180	1	2.0	0.517
	180	1—N_2 gas	2.0	0.557
	212	1	5.0	0.078
	212	1—N_2 gas	5.0	0.121

TABLE 3.—*Comparison of six methods of extraction using titrimetric peroxide procedure*

SAMPLE	BLANCH (°F.)	EXTRACTION PROCEDURE	ALIQUOT (ML)	AV. P.E. VALUE/MIN./G. TISSUE
2 (b) Guaiacol Procedure				
Pea pods	212	1	10.0	0.195
	212	2	10.0	0.092
	212	3	10.0	0.110
	212	4	10.0	0.258
	212	4	10.0	0.263
	212	5	10.0	0.111
	212	6	10.0	0.136
2 (a) Modified Balls and Hale Pyrogallol Procedure				
Pea pods	212	1	10.0	0.085
	212	2	10.0	0.105
	212	3	10.0	0.112
	212	4	10.0	0.105
	212	5	10.0	0.033
	212	6	10.0	0.056

Ascorbic Acid Oxidation Procedure. The data in Table 4, obtained by the ascorbic acid oxidation procedures 3a and 3b, show that both procedures are very good in sensitivity, simplicity, ease of duplication, and rapidity. Procedure 3a is better than 3b in these respects. These procedures are highly sensitive and therefore can be used on extracts of low peroxidase activity. The determination requires only a few minutes as compared to the greater lengths of time required by other methods.

Using procedure 3a for measuring the peroxidase activity, Table 5 shows that the citrate buffer extracts (methods 2 and 3) were of greatest activity. These were followed by the NaCl solution (method 1); then by methods 6 and 5, respectively. Townsley, however, suggested the use of NaCl as the extraction medium (method 1) in preference to the citrate buffer for this determination. When citrate is used the amount of 2,6-dichlorophenol indophenol used in the titration is affected by the time taken for the titration. The citrate seems to enter into the oxidation-reduction system in some way so that the end point will appear early in the titration. For example, if the solution is titrated not quite to the end point, the end point color will appear merely upon standing a few seconds.

Potassium Iodide Oxidation. Although the potassium iodide procedure (4) appeared to be the most promising of all the procedures examined, it was not found reliable. The time for the first color change between

TABLE 4.—*Ascorbic acid oxidation procedure*

SAMPLE	BLANCH (°F.)	EXTRACTION PROCEDURE	ALIQUOT (ML)	P.V./MIN./G. OF TISSUE
		Method 3a		
Asparagus	R.T.	1	0.04	4.310
	180	1	1.0	0.713
	212	1	1.0	0.093
Spinach	R.T.	1	0.1	10.843
	R.T.	1	0.04	9.644
	180	1	1.0	0.238
	212	1	1.0	0.119
Peas	R.T.	1	0.004	31.279
	180	1	1.0	0.383
	212	1	1.0	0.127
Pea pods	R.T.	1	0.04	26.360
	180	1	0.1	6.204
	212	1	0.1	2.403
		Method 3b		
Asparagus	R.T.	1	1.0	0.837
	180	1	2.0	0.042
	212	1	2.0	0.062
Spinach	R.T.	1	0.04	0.521
	R.T.	1	0.2	2.919
	R.T.	1	1.0	3.107
	180	1	2.0	0.042
	212	1	2.0	0.017
Peas	R.T.	1	0.2	12.564
	R.T.	1	0.1	13.137
	180	1	2.0	0.058
	212	1	2.0	0.034
Pea pods	R.T.	1	0.1	22.388
	180	1	0.1	4.275
	180	1	0.5	5.130
	180	1	0.4	5.422
	212	1	0.4	1.216

vegetables blanched at 212°F. and 180°F. was very close, and thus would not give a clear indication of the temperature or length of time of blanch under practical conditions. This does not disqualify the procedure but it is felt that further modifications of this test are necessary.

TABLE 5.—*Comparison of six methods of extraction, using ascorbic acid oxidation method 3a*

SAMPLE	BLANCH (°F.)	EXTRACTION PROCEDURE	ALIQUOT (ML)	P.T./MIN./G. OF TISSUE
Asparagus	R.T.	1	0.1	4.353
	R.T.	1	0.4	4.014
	R.T.	2	0.2	6.073
	R.T.	3	0.2	5.735
	R.T.	4	0.2	3.232
	R.T.	5	0.2	3.754
	—	—	—	—
	180	1	1.0	0.723
	180	2	1.0	2.516
	180	3	0.5	2.626
	180	4	1.0	0.411
	180	5	1.0	0.301
	180	6	1.0	0.298
Spinach	180	1	—	—
	180	2	1.0	0.373
	180	3	1.0	0.447
	180	4	1.0	0.140
	180	5	1.0	0.085
	180	6	1.0	0.111
Peas	R.T.	1	0.02	35.023
	R.T.	2	0.01	39.536
	R.T.	3	—	—
	R.T.	4	0.02	19.338
	R.T.	5	0.02	25.569
	R.T.	6	0.02	24.065
	180	1	1.0	0.467
	180	2	0.5	0.686
	180	3	0.5	0.734
	180	4	1.0	0.160
	180	5	1.0	0.192
	180	6	1.0	0.239
Pea pods	180	1	0.1	6.251
	180	2	0.05	17.576
	180	3	0.05	18.674
	180	4	0.1	2.040
	180	5	0.15	2.999
	180	6	0.15	3.261
	212	1	0.25	2.320
	212	2	0.10	6.455
	212	3	0.10	6.869
	212	4	0.50	0.642
	212	5	0.50	1.098
	212	6	0.50	1.353

COLLABORATIVE TRIALS ON LIMA BEANS

In view of the variability in results obtained at the first trial (11) and the difficulty of preparing and shipping replicate samples of frozen vegetables to collaborators (14), it was decided through conference with Dr. H. Olcott and his staff of the Vegetable Products Section of The Western Regional Research Laboratory to limit the collaborative work to four samples of Fordhook lima beans, to be ground while frozen, mixed and divided into two lots. Using the same reagents, one was to be analyzed by three collaborators at the Department of Food Technology, Berkeley, the other to be analyzed by three collaborators at Albany. (The collaborators were: M. A. Joslyn, A. Lukton, and C. J. B. Smit of the Department of Food Technology, University of California, Berkeley 4, California, and H. J. Morris, M. P. Masure, and S. Schwimmer of the Vegetable Products Section, Western Regional Research Laboratory, U. S. Department of Agriculture, Albany 6, California.)

The methods investigated were limited to three: the colorimetric guaiacol method, essentially as previously given as method 1b, the method of Morris, *et al.* (10), for carrot peroxidase, and the guaiacol titrimetric peroxidase procedure of Morris, *et al.* (10), a modification of that given as 2b. At the suggestion of Morris, the guaiacol-H_2O_2 concentrations were made the same as in 1b, and a 50 ml reaction flask was substituted for the 250 ml flask used previously. The addition of molybdate catalyst to H_2SO_4 was omitted because of the possibility that guaiacol would act as pyrogallol and phenol, which were found by Balls and Hale (15) to interfere. The period of standing of acidified KI-H_2O_2 solutions to obtain complete oxidation was taken as 20 minutes on the basis of Balls and Hale's (15) work on pyrogallol. The ascorbic acid oxidation procedure, 3a, was modified to meet Meuron's objection (see Joslyn (11)), by increasing the quantities used five-fold so as to obtain larger titration values. The procedures as modified are given below:

Extraction.—50 gram portions of frozen ground lima beans were comminuted in a Waring Blendor with 200 ml cold (precooled to 0°C.) 2% aqueous sodium chloride solution for 3 minutes, then filtered through a gauze backed cotton milk filter in a Büchner funnel by suction. 1 ml of enzyme extract was taken to represent 0.2 g tissue. Fordhook lima beans, (1) unscalded (raw), (2) scalded for 40 seconds, (3) scalded for 75 seconds, and (4) scalded for 105 seconds, were used. When 20 ml portions of the extracts were tested qualitatively by adding 1 ml of 0.75% H_2O_2 (catalase), and 1 ml of 0.75% H_2O_2 plus 1 ml of 10% guaiacol in 95% alcohol (peroxidase), they were found to give a strong catalase reaction and a fairly strong peroxidase reaction which varied as follows:

Sample	*Catalase*	*Peroxidase*
1 (raw)	5+	5+
2 (40 sec.)	5+	±
3 (75 sec.)	3+	±
4 (105 sec.)	+	−

The strong evolution of oxygen from H_2O_2 tended to obscure the guaiacol test even when observed immediately after mixing. On standing at room temperature for 30 minutes, the red color of the oxidized product of guaiacol bleached out.

1b. Colorimetric Guaiacol Procedure.—Prepare reaction mixture by adding 2.5 ml of 1 *M* acetate buffer at *p*H 5.6, 1 ml of 10% guaiacol in 95% alcohol, and sufficient water to make 50 ml when the enzyme extract (usually 1–5 ml, depending on activity) and 1 ml of 0.75% hydrogen peroxide (1 ml of 30% reagent diluted to 40 ml with cold distilled water) are added. Add the hydrogen peroxide last, after the mixture reaches room temperature (or 25°C.). Stir the completed mixture, pour a portion into a colorimeter tube for determination of the rate of color formation. Determine rate of color formation in Klett-Summerson colorimeter, using filter No. 42, recording values at approximately 30 second intervals over a 10 minute time period, and express activity as change in colorimeter reading per minute per gram of tissue.

2b. Titrimetric Guaiacol Procedure.—Bring to 25°C. 2.5 ml of 1 *M* acetate buffer (*p*H 5.6), 1 ml of 10% guaiacol in 95% alcohol, and sufficient water to make 50 ml when the enzyme extract (1–10 ml) and 1 ml of 0.75% H_2O_2 are added. Add the enzyme extract and hydrogen peroxide. Immediately after adding the H_2O_2, mix thoroughly and quickly remove a zero-time aliquot of the completed reaction mixture with a 10 ml rapid-flow pipet and blow it into a 125-ml Erlenmeyer flask containing 10 ml of 2 *N* sulfuric acid (55 ml conc. H_2SO_4 diluted to 1 liter). (Zero time is the time that delivery of the aliquot from the pipet is started.) Remove additional aliquots at 2.5, 5, and 7.5 minutes. At any time within an hour add to each flask 10 ml of sodium thiosulfate in 10% potassium iodide (100 g of KI, 2.5 g $Na_2S_2O_3 \cdot 5H_2O$, and about 1 g Na_2CO_3 dissolved in water and made up to 1 liter) and mix. After standing for 20 minutes, titrate excess thiosulfate with 0.01 *N* iodine, using about 20 drops of 1% starch as indicator. From the titrations calculate the peroxidase units as:

$$\text{P.E.} = \frac{a - x}{t}$$

where a = initial iodine equivalent of unchanged H_2O_2, and
x = iodine equivalent of residual H_2O_2 at time t.

3a. Ascorbic Acid Oxidation Procedure with Leuco 2,6-Dichlorobenzenone Indophenol as Substrate.—Pipette 5 ml of enzyme filtrate (for low enzyme activity blanched vegetables; dilute active preparations) into a 125 ml flask. Add 5 ml of ascorbic acid solution (50 mg of ascorbic acid dissolved in 100 ml of sodium oxalate-boric acid buffer. Prepare the buffer by dissolving 20 g of sodium oxalate in distilled water by gentle heating and continuous stirring. Make to 1 liter and add ca 20 g boric acid until *p*H 5.6 is reached, and filter). Then add 15 ml of 2,6-dichlorphenolindophenol solution (prepared by dissolving 200 mg of dye in 100 ml warm distilled water, filtering, and diluting to 1 liter at room temperature). Shake and transfer flask to constant temperature bath at 25°C. Swirl flask in water bath for 1 min, then remove from water bath, add 1.0 ml of 0.75% H_2O_2 from a blow-out pipette, and start stop watch. Shake flask and return to water bath. After exactly 1 minute, quickly add 5 ml of inhibiting acid (100 g metaphosphoric acid dissolved in cold water, filtered and brought to 1 liter). Titrate mixture with dye until a pink color is permanent for 30 seconds. Standardize dye solution against freshly prepared ascorbic acid (the dye solution should be stored in refrigerator and prepared weekly). Calculate the peroxidase value as number of mg of ascorbic acid oxidized in 1 minute by 1 g of enzyme-containing substance at 25°C.

$$\text{P.V.} = \frac{x \times c}{g}$$

x = dye equivalent (in ml) of ascorbic acid oxidized (= dye equivalent of 5 ml ascorbic acid—dye used for titration in ml plus dye added initially).
c = concentration of dye in mg of ascorbic acid per ml.
g = g of enzyme-containing substance present in aliquot tested.

RESULTS

Colorimetric Procedure.—In carrying out the colorimetric procedure at Berkeley, a 35% solution of hydrogen peroxide was diluted to 0.75% (0.86 ml diluted to 40 ml), and both a slightly oxidized guaiacol and a freshly distilled guaiacol were used. No difference was found between the two lots of guaiacol. The enzyme reaction mixture was prepared in a 50 ml volumetric flask at first. The mixture was brought to slightly less than 49 ml, then 1 ml of H_2O_2 added, the solution quickly made to volume, and vigorously shaken. Excessive frothing occurred, possibly accentuated by the evolution of O_2 from the mixture, and some time was lost in transferring the reaction mixture to the colorimeter tube. Subsequently the volumetric flasks were graduated at 49 ml and contents brought to 49 ml before addition of H_2O_2. This saved about 15–30 seconds but still resulted in excessively frothy mixtures. When 50 ml graduated cylinders were used less frothing occurred. At the time of preparing a test solution, a blank solution containing buffer, enzyme extract, and guaiacol, but without H_2O_2, was prepared to set the colorimeter scale to 0. Even with 2 ml of enzyme extract, however, the optical density of this blank was high (400–500 scale) so that the readings had to be taken at the portion of the scale which was less sensitive. Furthermore there was a considerable difference between the blank reading and the zero time reading. The enzyme suspension in the reaction mixture, particularly in the lima beans of lower residual peroxidase activity, coagulated during the readings, and the readings fell off with time because of this. At the suggestion of Masure we tried to use sufficient extract to give a difference in colorimeter readings (delta) of 60 per minute, but this was difficult to attain even with raw beans. Considerable variations were observed in the readings, and the results presented in Table 6 were calculated in two ways: (1) from the weighted average maximum delta colorimeter readings per 30 seconds over the range of 0–90 seconds, and (2) from average delta colorimeter readings in the range of 30–210 seconds. Duplicate results were difficult to obtain except for the scalded beans. Agreement between the three collaborators at Berkeley was only fair; two of the collaborators (Aaron Lukton and C. J. B. Smit) were fairly close together and the third (M. A. Joslyn) was high except for Sample II.

The data obtained on the same samples by the Albany group is shown in Table 7. The peroxidase values were expressed in two ways: (1) Activities based on initial reaction rates, and (2) activities based on averages over the first 10 minute period of reaction. Expression by the first method seems to be preferable since often the reaction had stopped before the 10

minute interval had elapsed; hence, an average over the entire 10 minute interval does not seem justified.

In general the results obtained by an individual collaborator for the more significant initial rate were as variable as those obtained by the Berkeley group. The difference between collaborators was quite appreciable, particularly for samples of higher enzyme activity.

TABLE 6.—*Delta colorimeter readings per 30 sec. and calculated peroxidase values*

(Berkeley group)

SAMPLE	ANALYST	ALIQUOT (ML)	Δ MAX	Δ AVERAGE PEROXIDASE VALUE		
			(1)	(2)	(1)	(2)
I (raw)	M.A.J.	2	30	22.5	150	112.5
		2	24	13.5	120	67.5
	A.L.	2	30	32.5	150	162.5
		2	31	31.3	155	156.5
		3	64	47	213.3	156.6
	C.S.	2	32	28.3	160	141.5
		2	30	28.0	150	140.0
II (40 sec.)	M.A.J.	3	22	12	73.3	40.0
	A.L.	2	16	12.7	80.0	63.5
		2	14	14.0	70.0	70.0
	C.S.	3	23	19	76.6	63.3
		3	29	21.5	80.0	71.7
		3	23.5	20	78.3	66.7
III (75 sec.)	M.A.J.	5	23.3	11	46.6	22.0
	A.L.	10	20	15	20.0	15.0
		10	20	15.5	20	15.5
	C.S.	10	19	13.3	19.0	13.3
		10	18	14.3	18.0	14.3
IV (105 sec.)	M.A.J.	5	3	1.5	6.0	3.0
	A.L.	10	0	0.0	0.0	0.0
	C.S.	10	1	0.0	0.5	0.0

(1) Delta colorimeter reading per 30 sec. in range 0–90 sec.
(2) Delta colorimeter reading per 30 sec. in range 30–120 sec.

Titrimetric Procedure.—In the titrimetric procedure particular care had to be taken to dilute the peroxide to 0.75%. The 10 ml of $Na_2S_2O_3$-KI solution contained just barely sufficient $Na_2S_2O_3$ to reduce the iodine liberated by 1 ml of H_2O_2. Variation occurred in the blank due to variation in delivery of H_2O_2 blown out of the 1 ml pipette. Duplicates were difficult to obtain in all cases, and the zero time titration varied apparently because of the loss of H_2O_2 due to the high catalase activity even of the scalded samples. The initial titration values varied from less than 0.5

to over 5.0 ml, increasing with increase in time of scalding and volume of sample used. The unscalded and partly scalded samples (I and II) gave more variable results than did the samples which were scalded more (III and IV). Sample II gave results appreciably higher than those found in sample I. Even in the raw lima beans duplication was not good. Slightly oxidized guaiacol gave results similar to those obtained with redistilled guaiacol and a 3% solution of H_2O_2 diluted to 0.75% gave results similar to those obtained with a diluted 35% solution.

The data obtained by the Berkeley group is summarized in Table 8.

The results obtained by the Albany group, expressed as moles of H_2O_2 used up per min. per gram of tissue, are shown in Table 9.

Duplicate determinations by this method checked quite well. However, it is difficult to explain why the 40-second blanched sample showed a greater activity than the raw sample.

TABLE 7.—*Delta colorimeter readings and calculated peroxidase values*

(Albany Group)

SAMPLE	ANALYST	ALIQUOT (ML)	VALUES[1]			
			INITIAL SLOPE[2]		AVERAGE[3]	
I (raw)	S.S.	1	66	59	57	54
		2	75	70	58	47
	M.P.M.	1	167	133	45	49
		2	180	139	54	48
	H.J.M.	1	125	110	37	29
		2	135	130	43	32
II (40 sec.)	S.S.	1	37	32	33	29
		2	33	29	9	8
	M.P.M.	1	62	49	16	12
		2	67	55	18	14
	H.J.M.	1	85	67	12	11
		2	90	50	16	10
III (75 sec.)	S.S.	1	7	4	4	2
		2	6	5	6	3
	M.P.M.	1	25	19	5	4
		2	21	15	6	4
	H.J.M.	1	20	14	3	3
		2	0	0	0	0
IV (10 sec.)	S.S.	1	0	0	0	0
		2	0	0	0	0
	M.P.M.	1	5	5	2	1
		2	—	—	—	—
	H.J.M.	1	0	0	0	0
		2	0	0	0	0

[1] Activities per minute per gram of tissue.
[2] From initial slope of delta colorimeter readings vs time.
[3] From average of delta colorimeter readings over ten minutes.

Table 8.—*Titrimetric guaiacol peroxidase values as K*

(Berkeley Group)

SAMPLE	ANALYST	ALIQUOT (ML)	K			NUMBER OF RUNS
			AVERAGE	MAX.	MIN.	
I	M.A.J.	1–4	0.65	0.87	0.49	7
	A.L.	2	0.63	0.96	0.47	8
	C.S.	2	0.53	0.56	0.49	4
II	M.A.J.	3	1.19			1
	A.L.	2–3	0.75	0.84	0.61	4
	C.S.	3	0.68	0.70	0.64	3
III	M.A.J.	5	0.73			1
	A.L.	4, 10	0.14	0.18	0.09	4
	C.S.	5, 10	0.19	0.23	0.09	8
IV	M.A.J.	5	0.82			1
	A.L.	10	0.07	0.11	0.04	4
	C.S.	10	0.11	0.24	0.04	3

Table 9.—*Titrimetric peroxidase values*

(Albany Group)

SAMPLE	ANALYST	ALIQUOT (ML)	MOLS OF H_2O_2 DECOMPOSED PER MIN. PER GRAM TISSUE ($\times 10^7$)[1]	
I (O)	S.S.	1	158	152
		2	155	145
	M.P.M.	1	195	187
		2	187	172
	H.J.M.	1	168	165
		2		
II (40)	S.S.	1	197	197
		2	184	176
	M.P.M.	1	225	218
		2	268	214
	H.J.M.	1	214	206
		2	223	216
III (75)	S.S.	1	117	109
		2	167	160
	M.P.M.	1	170	162
		2	167	158
	H.J.M.	1	170	160
		2	175	170
IV (105)	S.S.	1	77	77
		2	75	75
	M.P.M.	1	126	117
		2	114	111
	H.J.M.	1	117	117
		2	123	123
		2[2]	123	121

[1] From averages for periods 0 to 2.5 min. and 2.5–5 min.
[2] Assayed without guaiacol in mixture.

One of the collaborators (HJM) assayed the 105-second blanched sample, omitting guaiacol from the reaction mixture and found the same apparent activity as was observed when guaiacol was present. It is suggested that some "catalase type" activity may be present in the samples and may be responsible for the anomalies.

TABLE 10.—*Ascorbic acid oxidation procedure*

(Berkeley Group)

SAMPLE	ANALYST	ALIQUOT (ML)	DYE TITER	AA[1] DYE	BLANK	PV.[2]	P.V./G.[3]
I	M.A.J.	0.2	25.4	27.9	—	0.224	5.58
		0.5	22.1	27.9	—	0.520	5.20
	A.L.	0.5	22.03	27.73	27.53	0.512	5.12
	C.S.	0.25	25.49	28.10	28.2	0.232	4.64
		0.50	23.44	28.10	28.2	0.412	4.12
II	M.A.J.	0.50	26.5	27.9	—	0.126	1.26
		1.00	25.7	27.9	—	0.197	0.99
	A.L.	0.50	27.0	28.25	28.25	0.1106	1.11
	C.S.	1.0	26.53	28.14	28.48	0.143	0.72
III	M.A.J.	2.0	26.05	27.9	—	0.166	0.42
		5.0	24.25	27.9	—	0.327	0.33
	A.L.	2.0	27.2	28.38	28.45	0.104	0.26
	C.S.	2.0	27.03	28.21	28.71	0.1046	0.26
IV	M.A.J.	5.0	26.5	27.9	—	0.1254	0.13
	A.L.	2.0	28.24	28.35	30.13	0.0097	0.024
	C.S.	5.0	28.65	—	30.15	0.000	0.000

[1] Volume of dye solution equivalent to 5 ml of ascorbic acid buffer solution.
[2] Mg ascorbic acid oxidized per minute.
[3] Mg ascorbic acid oxidized per minute per gram vegetable tissue.

Ascorbic Acid Oxidation Procedure. This procedure, based on the method of Lucas and Bailey (16) for dehydrated vegetables, a modification of which they also proposed (17) as a quick test, was again found to give fairly reproducible duplicate results (Table 10). In this modification, the 15 ml of dye was added from a 25 ml buret to the reaction mixture and then after 1 minute the titration was completed with the same buret. However, the agreement between collaborators was not good. The results obtained by the Albany group are shown in Table 11.

One of the collaborators (HJM) has suggested a modification of this method. Results are expressed as described in the specified procedure, and also according to the suggested procedure. In the latter case a reagent blank is determined and applied in subsequent calculations. More details concerning this procedure will be furnished later.

TABLE 11.—*Ascorbic acid oxidation procedure*
(Albany Group)

SAMPLE	ANALYST	ALIQUOT (ML)	MG ASCORBIC ACID PER GRAM TISSUE PER MIN.			
I (0)	S.S.	1	5.4	5.1		
		2	5.7	5.1		
	M.P.M.	1	8.4	7.6		
		2	8.3	8.2	(b)	(b)
	H.J.M.	1	7.6	7.9	6.9	6.3
		2	8.7	7.5	7.5	6.4
II (40)	S.S.	1	1.5	1.4		
		2	1.3	1.3		
	M.P.M.	1	2.7	2.6		
		2	2.7	2.7		
	H.J.M.	1	1.6	1.4	1.8	1.4
		2	1.7	1.3	1.8	1.2
III (75)	S.S.	1	0.2	0.2		
		2	0.2	0.2		
	M.P.M.	1	0.25	0.23		
		2	0.31	0.27		
	H.J.M.	1	0.14	0.10	0.34	0.29
		2	0.10	0.10	0.33	0.30
IV (105)	S.S.	1	0.1	0.1		
		2	0.1	0.1		
	M.P.M.	1	+0.02	−0.02		
		2	+0.01	−0.02		
	H.J.M.	1	(a)	(a)	0.19	0.14
		2	(a)	(a)	0.15	0.13

(a) Less than zero activity when calculated in accordance with directions furnished collaborators.
(b) Data calculated in accordance with procedures suggested by H.J.M.

CONCLUSIONS

In view of previous results and those reported for the lima beans in 1952, it is recommended* that the ascorbic acid procedure, which appears to be less subject to error, be selected for further intensive study and that investigations of several factors (initial concentration of ascorbic acid, initial concentration of indophenol, initial concentration of H_2O_2, pH, temperature, time of reaction, and method of calculation of results) be carried out.

* For report of Subcommittee C and action of the Association, see *This Journal*, 36, 54 (1953).

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No report was given on quality factors; moisture in dried vegetables, or on catalase in frozen vegetables.

REPORT ON OILS, FATS, AND WAXES

BY G. KIRSTEN (Food & Drug Administration, Federal Security Agency, New York 14, N. Y.), *Referee*

No reports were made for 1952 by the Associate Referees on antioxidants, spectrophotometric methods of oil analysis, or quantitative methods for peanut oil. The Associate Referee has developed quantitative methods for the determination of butylated hydroxyanisole and nordihydroguaiaretic acid and plans to subject these methods to collaborative study during the coming year. He will also continue his study of proposed methods for other antioxidants. The Associate Referee for spectrophotometric methods has done some preliminary work and expects to have a report for the 1953 meeting.

RECOMMENDATIONS

It is recommended*—

(1) That studies on quantitative methods for peanut oil be continued.

(2) That studies on spectrophotometric methods for oils be continued.

(3) That collaborative work on methods for the determination of butylated hydroxyanisole and nordihydroguaiaretic acid be conducted by the Associate Referee.

* For report of Subcommittee C and action of the association, see *This Journal*, **36**, 58 (1953).

No reports were given on: spectrophotometric methods; peanut oil; antioxidants.

REPORT ON DAIRY PRODUCTS

By WILLIAM HORWITZ (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Referee*

Foreign Fats.—The regulatory necessity for the detection of the presence of foreign fats in butterfat has resulted in the appointment of a new Associate Referee who initially has undertaken to restudy the sterol acetate melting point methods, **26.33–.34.** This field has assumed such importance that a number of laboratories are studying the problem from many different points of view. Certainly the application of the modern tools of analytical chemistry such as chromatography, paper chromatography, infrared spectroscopy, and quantitative fatty acid separations may lead to methods of sufficient sensitivity to detect as little as 1 per cent foreign fat in butterfat. The present methods, such as the sterol acetate melting point, Reichert-Meissl and Kirschner values, and the critical temperature of dissolution are adequate to differentiate vegetable oil products from butterfat products and even to estimate the proportion of vegetable oil in mixtures containing substantial quantities of these foreign fats. The difficulty of detecting the addition of foreign fats at low levels can be appreciated from the fact that the methods in use today are the same as those used almost half a decade ago.

Babcock Test.—For several years the Associate Referee has recommended some substantial changes in the present procedure for the performance of the classical Babcock test for fat in milk, to bring the results closer to those obtained by the official Roese-Gottlieb procedure. The Referee, however, has felt that the possibility of the development of a detergent type test that would eliminate the use of sulfuric acid altogether was so promising that the adoption of such changes, although supported by collaborative work, should be delayed for a reasonable period of time. During the year the Bureau of Dairy Industry has announced such a test. The Referee recommends that this test be submitted promptly to collaborative study together with the modified test recommended by the Associate Referee to determine if it has the accuracy and precision necessary to warrant adoption by the Association.

Frozen Desserts.—The Associate Referee has developed a method for sucrose in ice cream. Five of his seven collaborators report results which are in good agreement for a polarimetric method. Two of the collaborators are out of line with the other results and therefore the Referee recommends further collaborative study. The Referee also recommends that further work be performed to perfect methods for fruits and nuts and that, if possible, collaborative work be performed on the titratable acidity method suggested by the Associate Referee.

Soft Cheeses.—The Associate Referee has demonstrated that there is a negligible loss of moisture during the preparation of sample of creamed cottage cheese by the Waring blendor and the Referee now recommends the adoption of this method as a procedure.

The expression as lactic acid of the results of the titratable acidity determinations on milk (**15.4**) and cheese (**15.129**) has been criticised on numerous occasions, since on sweet milk the measured titratable acidity is not due to lactic acid but rather to the natural buffers of the milk. The increase in acidity that occurs on souring is due chiefly to lactic acid, but the expression of all the results as lactic acid has become fixed in the terminology of the dairy industry. Scientifically, however, the results should be expressed as "ml 0.1 *N* NaOH per 100 g sample," and it is therefore recommended that this alternative terminology be added to these two methods.

Methods **15.40–.46** and **15.67**, Residual Phosphatase in Milk and Cream, are satisfactory for these products and have been in a first action status for a number of years. They should be adopted as official.

RECOMMENDATIONS*

It is recommended—

(1) That the method recommended by the Associate Referee be adopted as a procedure for the preparation of samples of creamed cottage cheese.

(2) That the following statement be added to the acidity methods for milk, **15.4**, and cheese, **15.129**, at the end of the next to the last sentences and be adopted as official:

"or as ml 0.1 *N* NaOH per 100 g sample."

(3) That methods **15.40–.46** and **15.67**, Residual Phosphatase in Milk and Cream, be adopted as official.

(4) That work on the following subjects be continued:

(a) Sucrose in ice cream and frozen desserts.

(b) Methods for the preparation of sample of frozen desserts which contain insoluble material such as fruit and nuts, etc.

(c) Titratable acidity in ice cream and frozen desserts.

(d) Mechanical shaking method for butter.

(e) Babcock method for fat in homogenized milk.

(f) Substitutes for sulfuric acid in the Babcock method for fat in dairy products.

(g) Detection and estimation of foreign fats in dairy products.

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* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 55 (1953).

REPORT ON FOREIGN FATS IN DAIRY PRODUCTS

THE STEROL ACETATE TEST

By J. H. Cannon (Food and Drug Administration, Federal Security Agency, St. Louis 1, Mo.), *Associate Referee*

The growing use of vegetable fats in frozen dessert foods and in other fatty food products gives increasing importance to tests for vegetable fat in mixtures with animal fat. The problem was considered by the A.O.A.C. in 1914 and 1915 (1), and *Methods of Analysis* contains the method which resulted from the 1914 work (2). It is based on the different physical properties of the sterols of vegetable and of animal fats. The melting point of cholesterol acetate is about 114°C. and that of phytosterol acetate is about 125–137°C. This test appears to be based on work done earlier by Bomer (3), Windaus (4), and others.

The collaborative work done in 1914 indicated that as little as 5 per cent of cottonseed oil can be detected in lard. The Associate Referee decided to try the method on butter adulterated with vegetable fats. Ten per cent of corn oil was readily detected. However, it was found that the method was not so successful when applied to a mixture of 10 per cent hydrogenated coconut oil and 90 per cent butter fat. This was probably due to the relatively smaller proportion of total sterol in coconut oil. Lewkowitsch says of the sterol acetate test: "The author has satisfied himself, by very exhaustive investigations carried out with a large number of oils and fats, as to the thorough reliability of this test. . . . A considerable number of other observers have also confirmed the reliability of this test; hence the opinion of a few chemists who found difficulties with this test may be disregarded as irrelevant" (5).

It is the purpose of the Associate Referee to adapt fractionation procedures to the present problem and to submit known mixtures of fats for collaborative study next year. Considerable preliminary work has already been done.

A procedure which has been successfully used to detect 5 per cent of coconut oil in 95 per cent of butterfat is given below:

DETECTION OF VEGETABLE FAT IN MILK FAT

DETERMINATION

Ext. fat from product to be tested. In general, 100 g of fat is required.* Saponify the 100 g fat by refluxing 2 hrs in a 2 l flask with 500 ml alcohol and 40 g KOH previously dissolved in 40 ml of H_2O.

Ext. the unsaponifiable matter as follows: Distil off most of the alcohol and dissolve residue in 1 l hot H_2O. Transfer to a 3 l separatory funnel. Cool somewhat and ext. with two 1 liter portions of ether, adding alcohol if necessary to facilitate

* The need for this size sample is apparent when one considers that a vegetable fat such as coconut oil containing only 0.08% sterol will yield only 4 mg. of sterol if present to the extent of 5% in the adulterated butter fat. This small amount must be separated, manipulated, and recrystallized.

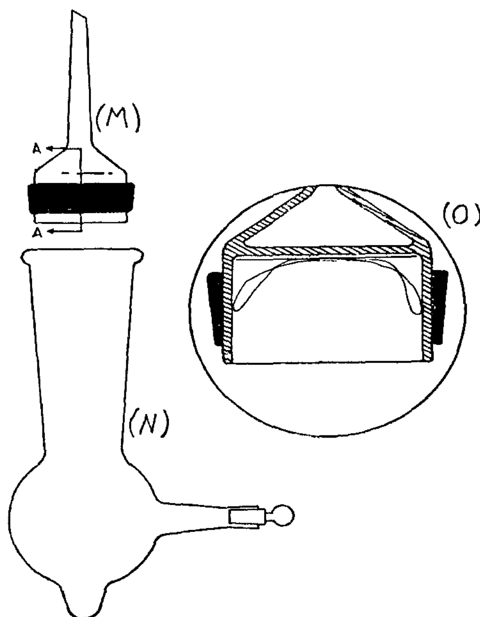

FIG. 1.—Special filter flask.

(M) Micro-Büchner funnel, Coors, size 0000, fitted with rubber collar.
(N) Flask, total height ca 125 mm, capacity of bulb ca 15 ml, ground-in stopper on side arm.
(O) Enlarged section at A-A showing springy wire retainer holding filter paper in place.

the sepn of the two liquids. Combine the ether exts and wash the ether soln first with 400 ml 0.4% NaOH soln, then with 400 ml 0.8% NaOH soln, and finally with 300 ml portions of H_2O until washings are not reddened by phenolphthalein. Evap. ether and dry the residue at 100°C.

Dissolve the unsaponifiable residue in a min. of hot methyl alcohol and transfer to special flask (Fig. 1). Chill in ice bath and filter with special filter. Return residue to special flask and again dissolve in min. of methyl alcohol. Chill and filter. Repeat this fractionation two or more times. Finally, dissolve the last residue in the special flask in methyl alcohol and filter thru a pledget of filter paper fiber into a 50 ml tared beaker. Evap., dry at 100°C., and weigh.

Add sufficient ethanol to dissolve the residue and add a hot 1% soln of digitonin, sufficient to provide about 1½ times as much digitonin as sterol. At this point, or on cooling, a heavy ppt of sterol digitonide separates. Add ether equal to the vol. of liquid in the beaker, stir thoroly and filter on a small fluted funnel covered with a watch glass during filtration. Rinse ppt and paper with several small portions of ether and allow ppt and paper to dry in a warm place (on a watch glass on top of steam bath). Carefully remove the digitonide from the paper with aid of a teasing needle. (The usual form of the ppt is very thin, papery, white sheets which readily come loose from the filter paper. They may be caught in a large watch glass as they fall from the paper.) Weigh the digitonide ppt after drying 15 min. at 100°C.

Transfer the dried digitonide ppt to the clean dry special flask and add 1–2 ml of acetic anhydride. (A suitable proportion of acetic anhydride to digitonide is 1 ml per 100 mg ppt.) Boil the acetic anhydride gently with a small heater for 15 min. (The ppt usually dissolves in 5–10 min.) Cool and add 10 ml 60% alcohol. Filter with special filter and return the ppt to the flask. Add 5 ml alcohol and heat to dissolve the ppt. Put pledget of filter paper pulp in orifice. Place flask on a support and aspirate air through soln until ppt forms. Filter through pledget and redissolve ppt in hot alcohol. Again aspirate until ppt forms and again filter. Repeat twice more if possible. When as many pptns as feasible have been made, dissolve final residue in hot alcohol and allow to filter through pledget, drop by drop, onto a hot, small (20 mm) watch glass sitting on a steam bath. As the last two drops are added to the watch glass, remove from the steam bath and, while observing with magnification, stir with a fine needle as the ppt forms, and as the mass becomes semisolid, scrape off the watch glass onto a piece of clean filter paper. Press another piece of filter paper down on the mass to remove adhering liquid. Finally, allow to dry for 10 min. at 100°C. Make melting point detn on this residue.

NOTES

Pure butter has never been found to yield a residue higher than 115.5°C. (Usually 114° to 115°C.) Authentic mixtures of hydrogenated coconut oil and butter have been found to yield melting points of from 118.5° for 5% coconut oil to 119.5° for 10% coconut oil when tested by the above procedure.

The special flask referred to in the above procedure is designed to facilitate the handling of the small precipitates involved. A diagram of the flask is shown below. The first filtrations are made on the Büchner funnel, the precipitate being scraped off the paper and returned to the flask each time. The final filtration through the pledget of filter paper pulp is made through the side arm, gentle air pressure being applied inside the flask through a rubber tube and rubber stopper to fit the mouth of the flask.

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REPORT ON FAT IN DAIRY PRODUCTS

VARIATIONS BY DIFFERENT TECHNICIANS IN ESTIMATING UPPER MENISCUS ON THE FAT COLUMN OF THE BABCOCK TEST FOR MILK

By ERNEST O. HERREID (Illinois Agricultural Experiment Station, Urbana, Ill.), *Associate Referee*

A number of investigators (1, 2, 3, 4, 7) have indicated the difficulty of determining the exact upper limits of the meniscus on the fat column of the Babcock test for milk. The purpose of this study was to determine variations among technicians in estimating the dimensions of the upper meniscus.

It was decided to have technicians in dairy plant laboratories, in state and in Federal regulatory laboratories, and in experiment station laboratories make Babcock fat tests on a number of samples of milk by standardized procedures.

PROCEDURE

After the fat tests were read by the regular Babcock method from their lower surface to the highest point of the upper meniscus, they were again placed in the water bath at 135 to 140°F. and held for 5 to 10 minutes. As each bottle was removed from the bath, two drops of a colored mineral oil (glymol) were added carefully to each bottle. The fat columns were again read from their lower surface to the fat-glymol line. It is assumed that the dimensions of the meniscus will be the difference between the readings with and without glymol. However, the lower end of the fat column does change slightly in shape as the bottles are removed from the water bath and exposed to room temperatures, but this will not greatly affect the dimensions of the upper meniscus, provided the tests are read quickly. Some of the technicians reported individual tests to 0.1 per cent while some others reported some of their tests to 0.05 per cent.

RESULTS

The dimensions of the upper meniscus on the fat column of the Babcock test as determined by 16 different technicians are given in Table 1. The

TABLE 1.—*The dimensions of the upper meniscus on the fat column of the Babcock test for milk as determined by different technicians*

TECHNICIAN	NUMBER OF TESTS	BABCOCK TEST		
		REGULAR	GLYMOL	MENISCUS
		Av. per cent	*Av. per cent*	*Av. per cent*
1[a]	81	4.35	4.21	0.14
2[a]	44	4.16	4.01	0.15
3[a]	88	4.26	4.12	0.14
4[a]	236	4.00	3.83	0.17
5[a]	48	4.20	4.00	0.20
6[a]	72	3.41	3.36	0.05
7[b]	96	3.95	3.90	0.05
8[b]	120	3.44	3.35	0.09
9[b]	72	5.05	4.93	0.12
10[b]	48	3.30	2.15	0.15
11[b]	96	3.74	3.63	0.11
12[b]	86	3.65	3.45	0.20
13[b]	96	3.64	3.44	0.20
14[b]	88	4.65	4.56	0.09
15[b]	55	3.60	3.42	0.18
16[c]	100	4.56	4.43	0.13
	1426			

[a] Experiment station laboratory.
[b] Commercial dairy laboratory.
[c] Regulatory laboratory.

results for technicians 1, 2, and 3 have been published (5) and are included because it is believed that they more nearly represent the actual dimensions of the meniscus which can be used as a basis for comparing results obtained by the other technicians. Also these results agree closely with those reported about 30 years ago by Phillips (6), who obtained 0.146 per cent for the meniscus. Technician 4 tested the largest number of samples and his values for the meniscus exceeded those obtained by technicians 1, 2, and 3, and his Babcock tests exceeded his Mojonnier tests by amounts greater than those of technicians 1, 2, and 3. Technicians 4, 5, 12, 13, and 15 obtained the highest results for the meniscus while the results obtained by technicians 6, 7, 8, 11, and 14 are considerably lower compared to the standard set by technicians 1, 2, and 3.

CONCLUSIONS

The results (Table 1) of 16 technicians who tested 1426 samples of milk indicate significant variations in estimating the dimensions of the upper meniscus on the fat column of the Babcock test.

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REPORT ON FAT IN HOMOGENIZED MILK

A MODIFIED BABCOCK METHOD

By C. E. Hynds (State Food Laboratory, Department of Agriculture and Markets, Albany, N. Y.), *Associate Referee*

A modified Babcock method for homogenized milk, with minimal changes from the standard procedure, was proposed and sent to several collaborators. Instructions were given to test in duplicate several samples of homogenized milk obtained locally and to compare the results with the Roese-Gottlieb and with the standard Babcock method. The proposed method is as follows:

PROCEDURE

Prepare sample and measure into test bottle as directed in standard Babcock method. Adjust milk and acid to 60–62°F. Add 17.0 ml H_2SO_4, sp. gr. 1.823–1.827 at

20°C. Shake in usual manner, mechanically or manually, until all curd is dissolved; then *shake continuously* for an addnl 5 min. Centrifuge for 8 min. Add ca one-half the amount of H_2O usually added at this point and *shake again* for 1 min. Add the remainder of the water to bring the level to the base of the neck. Complete the procedure and read as with the standard Babcock method.

RESULTS

Figures and comments were received from 4 laboratories. Laboratory No. 1 reported acid volume had to be increased to 22 or 23 ml. to obtain a clear fat column and suggested use of a higher milk and acid temperature. They commented, "If one uses the larger H_2SO_4 volume and discounts the extra time and manipulation required, suggested method is far superior to standard Babcock and somewhat better than the modification of Lucas and Trout." Laboratory No. 2 felt that the modified method they are using takes less time, and they reported closer agreement with the Roese-Gottlieb method than when the proposed modification was used. Laboratory No. 3 reported that the method seemed entirely satisfactory, and had no adverse comment. Their results showed very close agreement with the Roese-Gottlieb method. Laboratory No. 4 gave no comment except that their own modification seemed very satisfactory.

The following table summarizes the results from the 4 laboratories:

TABLE 1.—*Comparisons of fat determinations*

(Percentages of variance from determinations by Roese-Gottlieb method)

LAB. NO.	NO. OF SAMPLES	PROPOSED METHOD		STANDARD BABCOCK METHOD		MODIFIED METHOD AS USED IN VARIOUS LABORATORIES	
		GREATEST VARIATION	AVERAGE	GREATEST VARIATION	AVERAGE	GREATEST VARIATION	AVERAGE
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	13	−.09 to +.03	−.02	−.17 to +.18	+.05	−.11 to +.11	−.03
2	21	−.189 to +.017	−.072	−.260 to +.028	−.09	−.142 to +.045	−.037
3	9	−.035 to +.025	+.0016	−.265 to −.145	−.210	—	—
4	10	−.19 to .00	−.077	—	—	−.10 to .00	−.047

The modified method as used by the several laboratories is essentially the same as the standard Babcock method except that the acid is added in either 3 or 4 portions, the first portion being $\frac{1}{3}$ or $\frac{1}{2}$ of the total (17.5 ml) and the remainder in 2 or 3 equal amounts. After each addition, mixtures are shaken 30 to 45 seconds and one laboratory shakes mixture for 5 minutes after all acid is added. The first centrifuging is increased to 10 minutes and the test finished as in the standard procedure.

RECOMMENDATIONS

It is recommended* that studies be continued to discover the best portionwise procedure for adding the acid and shaking the samples.

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 55 (1953).

REPORT ON PREPARATION OF SAMPLE OF CREAMED COTTAGE CHEESE WITH THE WARING BLENDOR

By Sam H. Perlmutter (Food and Drug Administration, Federal Security Agency, Minneapolis 1, Minn.), *Associate Referee*

The Waring Blendor has proved to be an effective, rapid instrument for the comminution of creamed cottage cheese to a smooth uniform product. It has been suggested, however, that the heat generated during blending might cause considerable moisture loss. This study was made to determine the magnitude of such possible moisture losses.

A previous report (1) indicated that the replicate determinations on a large number of samples of creamed cottage cheese agreed very well after mixing with the Waring Blendor. The work done in this study further confirms this.

Naimark and Mosher (2) list the following disadvantages of the Waring Blendor: (1) heat generated during blending, (2) surface denaturation of proteins, (3) oxidation of the blended substances, and (4) copper contamination from worn blendor parts.

Since creamed cottage cheese becomes homogenous within five minutes, and since the most important disadvantage seemed to be the possible moisture loss through heating, the study was confined to this phase.

EXPERIMENTAL

A torsion balance sensitive to 0.1 g and with sufficient capacity to handle a loaded blendor jar was used in this study.

Experiment 1. One package of creamed cottage cheese (12 oz.) was put into the blendor together with a thermometer attached to the inside wall by means of cellophane sealing tape. This loaded jar was weighed and the temperature recorded. After mixing for one and one-half minutes, which was the time required for homo-

Table 1.—*Weight loss and temperature rise in a sample of creamed cottage cheese using an uncovered Waring Blendor for mixing*

Weight of cheese in sample: 339.1 grams
Initial temperature: 16.5°C.

NUMBER OF MIXINGS	TIME OF MIXING	TEMPERATURE	CUMULATIVE WEIGHT LOSS
	minutes	°C.	*grams*
Initial	1.5	19.5	0.1
2	2	23	0.1
3	2	25.5	0.1
4	2	31	0.3
5	2	32.5	0.4
6	2	36	0.3
7	2	37.5	0.5
8	2	42	0.7
9	2	44.5	0.8

geneity, the blendor was again weighed and the temperature of the contents recorded. No cover was used in these experiments. This operation was repeated at two minute intervals, time and temperature recorded as before, allowing one minute between runs for weighing and recording. The results are given in Table 1.

Experiment 2. Two 12-oz. packages of creamed cottage cheese were placed in a four l stainless steel beaker and mixed for five minutes with a large spoon and spatula. The cheese appeared adequately mixed. Five subdivisions were analyzed for moisture using the official vacuum oven method (3) but with only one weighing after four hours drying. Contents of beaker were then transferred to the Waring Blendor and mixed for two and one-half minutes. The sample was smooth and uniform. Five subdivisions were again analyzed for moisture as above. The results are given in Table 2.

TABLE 2.—*Comparison of the moisture values obtained by two different methods of mixing creamed cottage cheese; dried for 4 hours*

SUBDIVISION NUMBER	METHOD OF MIXING	
	SPOON AND SPATULA PER CENT MOISTURE	WARING BLENDOR PER CENT MOISTURE
1	78.38	78.68
2	78.27	78.65
3	78.44	78.65
4	78.65	78.71
5	78.55	78.72
Average	78.46	78.68

In this case a higher moisture value was obtained when the sample was prepared with the blendor. The coarser curds in the hand mixed sample may have entrapped the moisture and required a longer drying period.

Experiment 3. Two packages of creamed cottage cheese were placed in the Waring blendor together with a long handled teaspoon and the thermometer. The spoon was used to mix the cheese during blending to reduce the possibility of channeling with such a large load. Otherwise this experiment was conducted as in experiment 1. The results are given in Table 3.

TABLE 3.—*Weight loss in a sample of creamed cottage cheese using a Waring blendor for mixing and spooning the cheese into the blades to prevent channeling*

Weight of cheese: 666.4 grams
Initial temperature: 13°C.

NUMBER OF MIXINGS	TIME OF MIXING	TEMPERATURE	CUMULATIVE WEIGHT LOSS*
	minutes	°C.	*grams*
Initial	2	17	0.0
2	2	22	0.2
3	2	28	0.4
4	2	32	0.7

* The blendor developed a leak around the bearing during this experiment.

Experiment 4. Experiment 3 was repeated, using a spoon to reduce channeling. The results are tabulated in Table 4.

TABLE 4.—*Weight loss in a sample of creamed cottage cheese using a Waring blendor for mixing and spooning the cheese into the blades to prevent channeling*

Weight of cheese 657.4 grams
Initial temperature 20°C.

NUMBER OF MIXINGS	TIME OF MIXING	TEMPERATURE	TOTAL WEIGHT LOSS
	minutes	°*C.*	*grams*
Initial	2	27	0.2
2	2	32	0.4
2	2	—	0.6

Experiment 5. Two packages of creamed cottage cheese were placed in a stainless steel beaker and mixed by hand with a spoon and spatula for five minutes. The sample appeared adequately mixed. Five subdivisions were analyzed for moisture by the official vacuum oven method to constant weight (3). The contents of the beaker were transferred to the Waring Blendor and mixed until homogeneous (three minutes). Five subdivisions were analyzed for moisture. The results are tabulated in Table 5.

TABLE 5.—*Comparison of the moisture values obtained by two different methods of mixing creamed cottage cheese; dried to constant weight*

	METHOD OF MIXING			
	SPOON AND SPATULA PER CENT MOISTURE		WARING BLENDOR PER CENT MOISTURE	
	3 HRS. DRYING	1 HR. ADDL.	3 HRS. DRYING	1 HR. ADDL.
	76.42	76.52	76.52	76.57
	76.64	76.71	76.54	76.58
	76.37	76.47	76.59	76.63
	76.38	76.45	76.53	76.58
	76.63	76.70	76.57	76.62
Average	76.49	76.57	76.55	76.60

Experiment 6. This experiment is the same as experiment 5 except that the two packages of cottage cheese were allowed to remain uncovered in the refrigerator for one week before analysis to determine if surface drying of the curd affected the preparation of the sample. The results are given in Table 6.

SUMMARY

There is a slight but negligible loss in weight when cottage cheese samples are mixed in a Waring Blendor for the length of time necessary to produce a homogeneous, smooth sample. This loss did not exceed 0.06

TABLE 6.—*Comparison of the moisture values of creamed cottage cheese which had been standing uncovered in refrigerator one week, mixed by 2 different methods; dried to constant weight*

	METHOD OF MIXING			
	SPOON AND SPATULA PER CENT MOISTURE		WARING BLENDOR PER CENT MOISTURE	
	4 HRS. DRYING	1 HR. ADDL.	4 HRS. DRYING	1 HR. ADDL.
	75.76	75.79	75.73	75.74
	75.61	75.65	75.72	75.74
	75.58	75.65	75.76	75.77
	75.81	75.83	75.71	75.73
	75.74	75.77	75.74	75.76
Average	75.70	75.74	75.73	75.75

per cent in four minutes, which was sufficient time for mixing the samples examined in this study.

RECOMMENDATIONS

It is recommended* that the following method for the preparation of samples of creamed cottage cheese be adopted as a procedure:

Place 200–600 g sample at ca 15° in the quart cup of a high speed blendor and blend for the min. time (2 to 5 min.) required to obtain a homogeneous mixt. The final temp. should not exceed 25°. This may require stopping the blendor frequently after channeling and spooning the cheese back into the blades until the blending action starts.

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REPORT ON FROZEN DESSERTS

PREPARATION OF SAMPLE, SUCROSE, AND ACIDITY IN ICE CREAM

By HUGH M. BOGGS (Food and Drug Administration, Federal Security Agency, Philadelphia 6, Pa.), *Associate Referee*

SUCROSE IN ICE CREAM

A method for sucrose in ice cream, along the lines suggested in the Associate Referee's 1951 Report on Frozen Desserts (1), was developed and submitted to collaborative work during 1952. The method was along classical lines of clearing with neutral lead acetate, deleading with potas-

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 55 (1953).

sium oxalate, inverting with invertase* and reading the polarization before and after inversion on a standard saccharimeter. Values obtained are substituted in formula given in *Methods of Analysis*, 7th Ed., **15.90, and** corrections are made for the volumes occupied by fat and protein.

Results of five out of seven collaborators were in excellent agreement, but the results of the other two collaborators differed by more than 1 per cent from the average, which was quite near the expected result. Further work will be done on this method.

TABLE 1.—*Collaborative results for sucrose in ice cream*

COLLABORATOR	TEMPERATURE	DIRECT READING	INVERT READING (75 ML)	PER CENT SUCROSE FOUND	AVERAGE
A	32°	+23.2°	+4.4°	13.11	13.26
		23.2	4.1	13.41	
B	30°	25.5	4.2	14.91	14.86
		25.5	4.3	14.83	
		25.6	4.5	14.67	
		25.5	4.4	14.67	
		25.7	4.4	14.83	
		25.9	4.4	14.98	
		26.2	4.6	15.05	
C	27°	24.34	3.94	14.14	14.12
		24.48	4.18	14.00	
		24.54	4.22	14.01	
		24.68	4.00	14.33	
D	22°	22.36	0.08	16.18	16.22
		22.34	−0.08	16.28	
E	20°	25.02	3.96	14.22	14.21
		25.02	3.94	14.23	
		25.0	3.98	14.19	
		25.0	3.98	14.19	
F	24°	26.44	4.30	15.15	14.60
		24.44	3.92	14.05	
G	25°	24.75	3.33	14.96	14.69
		24.20	3.43	14.46	
		24.67	3.58	14.66	
		Average		14.57	14.57

* Brown (6) states: "Invertase inversion is unquestionably the best of the Clerget modifications" (6, 7). Bates agrees: "Of the two commonly employed hydrolytic agents, invertase is superior because of its highly selective action on the sucrose group, and because it is without effect on the rotation of other substances occurring in sugar samples" (8). However he goes on to list advantages and disadvantages of both invertase and hydrochloric acid as hydrolytic agents for sucrose.

PREPARATION OF SAMPLE

In the 1951 Associate Referee's Report (1) an alternative method for the preparation of samples containing insoluble particles of fruit, nuts, or confectionery, was recommended. The method was to remove the insoluble particles from the melted samples on a 30 mesh sieve, weigh them, and proceed to analyze that portion of the sample which passed through the screen and which approximates the original ice cream mix.

Objections have been raised to the thoroughness of this separation. It is conceivable that small particles of nuts with a high fat content may pass through the screen and give a high result for milk fat. Also a considerable amount of juice from soft fruits like berries or peaches goes through the screen. In fact, the amount of fruit retained is as dependent on the degree of comminution and the nature of the fruit as on the amount of fruit originally added.

Three samples of strawberry ice cream were examined by this method. All had been made by adding 25 per cent of 1+3 frozen fruits to the same basic mix. Each, therefore, contained 18.75 per cent of fruit. The fruit recovered amounted to 8.1, 13.0, and 10.0 per cent.

This examination was made by pouring the melted pint sample through the 30 mesh sieve, allowing to drain two minutes, and then washing with between a liter and two liters of water from a wash bottle with non-constricted tip. The fruit was placed on a paper towel by inverting the sieve over it and scraping out adhering particles, blotted lightly with another paper towel, collecting in a weighing scoop, and weighing.

If the limitations of the method are thoroughly understood, it is believed that inclusion in *Methods of Analysis* will serve a useful purpose.

ACIDITY IN FROZEN DESSERTS

Sommers and Minos (3) and Kruisheer (4) have reviewed quite extensively the literature on the subject of "acidity of milk." Milk is a good buffer, and Sommers and Minos believe this is largely due to the precipitation of tri-calcium phosphate when alkali is added. Dilution decreases this effect and also decreases the "salt effect" and "protein effect" so that a lower titration is obtained. The amount of phenolphthalein indicator used in the titration is also important, and as the amount used is decreased below 0.5 ml of 1 per cent solution, an increase in the apparent acidity of the sample occurs.

Johnson and King (5), after considerable study, recommend titration of the acidity in milk by comparison with a standard of the same milk to which has been added 1 ml of a 0.00024 per cent solution of rosaniline acetate, using 1 ml of 0.5 per cent phenolphthalein as indicator in the sample. They find, however, that this procedure is only slightly more desirable than titration to the "first perceptible color." It is believed

that the comparison procedure is impracticable for colored frozen desserts because of the colors already present.

In the method for acidity in milk (*Methods of Analysis*, 7th Ed. (1950) **15.4**) the sample is diluted with an equal volume of freshly boiled, distilled water and titrated with 0.1 N alkali, using 0.5 ml of 1 per cent phenolphthalein as an indicator. Titration to "first perceptible color" is implied. By using magnetic stirring and greater dilutions, if necessary, it is possible to detect an end point even in the most highly colored frozen dessert samples.

It is true that results on these colored solutions are somewhat higher, in general, than those on plain vanilla ice cream, but many of these products contain fruit, and ices will probably have a certain acidity from fruit juice or added food acids.

The statement of Johnson and King (5), that "The 'titratable acidity' in milk is an empirical value, having no exact equivalent in terms of a given acid (although usually expressed as lactic acid)" is even more true for frozen desserts. Nevertheless, this value serves a useful purpose.

RECOMMENDATIONS

It is recommended*—

1. That further work be done on the methods:
   (a) for sucrose in ice cream
   (b) for titratable acidity in ice cream and ice cream mixes
   (c) on the procedure for preparation of sample of frozen desserts containing insoluble particles.
2. That the method for sucrose in ice cream, with slight revisions, be submitted to further collaborative work.
3. That a method for titratable acidity in ice cream based on **15.4** be submitted to collaborative work, and that the results for titratable acidity be expressed as ml of 0.1 N alkali/100 g of sample, since expressing them as per cent lactic acid is misleading.

The Referee wishes to thank the following collaborators for their cooperation:

Glen C. Mowery and A. L. Amott, Division of Foods, Drugs, and Dairies, Tennessee Department of Agriculture; H. S. Peckinpaugh and L. B. Roberts, Alabama Department of Agriculture and Industries; N. E. Yongue and John A. Vignau, Department of Health, Washington, D. C.; Ernest S. Windham, Army Medical Graduate School, Walter Reed Army Medical Center; A. H. Robertson and Alice Waterhouse, State Food Laboratories, New York Department of Agriculture; and Ara C. Call and J. M. Stringham, Western Condensing Company, Appleton, Wisconsin.

Thanks are also due Carlton J. Austin and George Rodgers, of the Supplee-Wills-Jones Milk Co., Philadelphia, Pa., for their kindness in preparing and packing the samples.

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 55 (1953).

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No reports were given on: phosphatase test in dairy products; sampling, fat, and moisture in hard cheeses; preparation of butter samples; tests for reconstituted milk; and cryoscopy of milk.

The contributed papers, "Water Insoluble Acids and Butyric Acid in Cream Stored at 4°C.," by Fred Hillig and W. R. North, and "Effect of Feed on the Water-Insoluble Fatty Acids in Cream," by Fred Hillig and J. C. Palmer, were published in *This Journal*, November, 1952, pages 844 and 852, respectively.

The contributed papers, "Preparation of Sample of Pressurized Cream," by C. G. Cunningham, and "The Determination of Moisture in Process American Cheese and Process Cheese Food," by J. H. Cook H. C. Follstad, and W. W. Fisher were published in *This Journal*, February, 1953, pages 128 and 132, respectively.

"Ash in Non-Fat Dry Milk Solids" appears as a note on page 557.

REPORT ON EGGS AND EGG PRODUCTS

By F. J. McNALL (Food and Drug Administration, Federal Security Agency, Cincinnati 2, Ohio), *Referee*

ADDED GLYCEROL IN EGGS

As recommended by Subcommittee C, Associate Referee George E. Keppel has continued the study of a quantitative determination of glycerol in eggs, and has revised the present first action method, *This Journal*, **33**, 49 (1950), to make it applicable to the determination of glycerol in eggs containing added sugars.

In the proposed method the sugars are removed by treating with calcium oxide and alcohol, after preliminary removal of the protein and fat from an aqueous suspension of the egg mixture. The method has been studied collaboratively; the results are in good agreement and show adequate recovery of the added glycerol.

The Associate Referee has recommended that the proposed method for glycerol in the presence of added sugar be adopted as first action, and that the present method, **16.27** and **16.28**, be revised to include the proposed method. He also recommends that the subject be closed.

The Referee concurs in these recommendations.*

AMMONIA NITROGEN

No report was received from the Associate Referee assigned to the above subject. We do not believe that there is sufficient interest in this subject to recommend its continuance and therefore recommend that this study be dropped.

REPORT ON ADDED GLYCEROL IN EGG MIXTURES CONTAINING SUGARS

By George E. Keppel, (Food and Drug Administration, Federal Security Agency, Minneapolis 1, Minn.), *Associate Referee*

At the time the present method for added glycerol in eggs was adopted, Subcommittee C recommended that further work be done on the quantitative determination of glycerol in mixtures of eggs and sugars (1).

The proposed method, as finally developed, is an extension of the present method for added glycerol, in that an extra clarification step is provided to remove sugars. The sugar removal is based on a technique of Elving and his co-workers as used in a method (2) for glycerol in fermentation residues.

In the proposed method an aqueous suspension of the egg mixture is treated with sodium tungstate and dilute sulfuric acid to remove proteins and fats. An aliquot of the filtrate is further treated with calcium oxide and alcohol to remove sugars. After removal of alcohol, the solution is heated with sodium hydroxide to destroy remaining traces of sugars. The resulting solution, essentially free from interfering substances, is oxidized with potassium periodate, and glycerol is determined by titration of the resulting formic acid.

EXPERIMENTAL

Optimum conditions for removal of sugar interferences were developed using aqueous solutions of glycerol, sucrose, and dextrose. It was found that sucrose, in amounts up to 200 mg (equivalent to 50 per cent sucrose in a 2 g sample) caused no interference. With dextrose solutions, amounts up to 40 mg caused no interference. With 100 mg quantities, 1.6 per cent of the dextrose remained after the lime-alcohol-NaOH treatment. This figure was obtained by oxidizing an aliquot containing a known amount of dextrose with periodate, and titrating the formic acid obtained. From

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 55 (1953).

this titer and that of the treated dextrose solution, the per cent of dextrose remaining was calculated.

COLLABORATIVE STUDY

An egg mixture was prepared which consisted of fresh whole egg, U.S.P. glycerol of known strength, and 4.9 per cent each of sucrose and of dextrose. The mixture contained 8.0 per cent by weight of glycerol. After thorough mixing in a closed container, portions of the preparation were placed in 4 oz. jars, frozen, packed in dry ice, and sent to the collaborators. Duplicate determinations were requested.*

COLLABORATIVE RESULTS

Results obtained by collaborators are shown in Table 1.

TABLE 1.—*Collaborative results on added glycerol in egg mixture with sugars*

COLLABORATOR	GLYCEROL FOUND	RECOVERY
	per cent	*per cent*
1	8.01	100.1
	8.12	101.5
2	8.30	103.8
	8.30	103.8
3	7.90	98.8
	7.83	97.9
4	7.77	97.1
	7.89	98.6
	7.84	98.0

None of the collaborators reported difficulty in following the method. It was suggested that the alcoholic filtrate could be evaporated by boiling on an electric hot plate to reduce the time of analysis. This point was considered during development of the method, but experiments indicated a definite loss of glycerol if the filtrate was allowed to boil. The results obtained by the collaborators show satisfactory recoveries of the added glycerol.

RECOMMENDATIONS

It is recommended† that the proposed method for glycerol in the presence of sugars be adopted, as first action, after making the following changes in the present method:

* For the method, as adopted by the Association, see Changes in Methods, *This Journal*, **36**, 78, paragraph (b) (1953).
† For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 55 (1953).

16.27. Omit "(Not applicable in presence of added sugars)".

16.27. Add the following to list of reagents. "(d) Calcium oxide, powdered."

16.28. Before beginning of first line insert the following: "(a) Eggs with no added sugars."

New paragraph. "(**b**). Eggs containing added sugars." (Followed by method as given above, beginning "Prepare sample solution as directed in **16.28(a)** . . . ."

It is further recommended that the subject be closed.

The Associate Referee wishes to express his appreciation for the kind cooperation of the following collaborators, all members of the U. S. Food and Drug Administration: Richard F. Heuermann, St. Louis, Janice C. Bloomingdale, Chicago, and Juanita E. Breit, Minneapolis.

REFERENCES

(1) *This Journal*, **32**, 53 (1949).

(2) Elving, Philip J., Warshowsky, B., Shoemaker, E., and Margolit, J., *Anal. Chem.*, **20**, 25 (1948).

No report was given on Ammonia Nitrogen.

REPORT ON FEEDING STUFFS

By M. P. Etheredge (Mississippi State Chemical Laboratory, State College, Miss.), *Referee*

It is recommended*—

(1) That the study of the following subject be discontinued:

(a) Tankage (hide, hoof, horn, and hair content)

(2) That the work on the following be continued:

(a) Fat in fish meal

(b) Crude fat or ether extract

(c) Mineral constituents of mixed feed

(d) Drugs in feeds

(e) Crude protein in feeding stuffs

(f) Ash in feeding stuffs

(g) Milk by-products in mixed feeds

(h) Microscopic examination

(3) That the method for enheptin (2-amino-5-nitrothiazole), with alterations as outlined, be resubmitted for collaborative study.

(4) That collaborative studies of a method for nitrophenide (m,m'-dinitrodiphenyl-disulfide) be continued.

(5) That the method for sulfaquinoxaline, adopted as first action in 1949, be made official.

(6) That the method for the determination of cobalt in mineral feeds, adopted as first action last year, be made official.

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 48 (1953)

(7) That the method for crude fat in baked dog food as outlined by the Associate Referee be adopted, first action.

REPORT ON ASH IN FEEDING STUFFS

By R. L. Willis (New Jersey Agricultural Experiment Station, New Brunswick, N.J.), *Associate Referee*

It was brought to the attention of the Referee on feeding stuffs by the chief chemist of a large feed manufacturing concern that further work on the determination of ash should be carried on. The contention was that certain classes of feed materials and samples of ground pelletized mixed feed were difficult to ash in the prescribed time as set forth in our official methods. Your Associate Referee was directed to contact the above mentioned chemist with a view to preliminary investigation and collaboration between the two laboratories for the first year and, if conditions warranted, to submit samples to collaborators for study the following year. In accordance with this suggestion five samples of broiler mash were used in this study, and with the following results:

(1) By using a silica ashing dish (Fisher, catalog #8–112) check results were obtained by our two laboratories to within 0.02 per cent to 0.1 per cent. Results obtained by using a tall form silica crucible gave check results differing as much as two or more per cent. In five per cent or more of the determinations made in the taller form crucible, appreciable amounts of carbon were present.

(2) It was also noted in this collaborative study that when two or three samples were ashed in a muffle furnace of 20–30 crucible capacity, the results invariably averaged 0.5 per cent lower than when the muffle was loaded to capacity.

(3) The results obtained by ashing in Coors crucibles (tall form) were approximately 0.5 per cent higher than those ashed in the silica dish.

After studying the reports of Dr. St. John on the subject of ash* and noting the many angles investigated in this study, your Associate Referee fails to find any part that could basically be changed or improved upon. The collaborative work of the two laboratories noted is insufficient to justify any recommendation other than that this study be continued for the purpose of establishing a standard crucible for ash determination.

REPORT ON COPPER DETERMINATION IN MINERAL FEEDS

By J. C. Edwards (Department of Agriculture, Tallahassee, Fla.), *Associate Referee*

Copper was selected for study this year since there is no official method for the determination of this element in mineral feeds. A commercial

* See *This Journal*, **22**, 628 (1939); **23**, 620 (1940); **24**, 848 (1941); **25**, 857 (1942).

mineral feed sample was picked at random, reground, and remixed; portions were cut out and mailed to the several collaborators. It was felt that this kind of sample would present most difficulties likely to be encountered with the method under study. In addition to copper, the sample contained approximate percentages of the following: Ca 13.8, P 3.6, Co 0.029, Mn 0.044, Fe 0.21, NaCl 35.0. None of these elements offered any serious interference.

RESULTS

Collaborator	*Per cent Cu*	*Per cent Cu by Other Methods*
1	0.42	
2	0.43	
3	0.50	
4	0.43	
5	0.44	
6	0.53	
7	0.32*	0.48 Sodium Diethyldithiocarbamate
8	0.48	
9	0.46	
10	0.53	
11	0.44	
12	0.48	
13	0.53	0.52 A.O.A.C. Method 24.23 (Omitting KI Stripping Solution) and determination of Copper by Sodium Diethyldithiocarbamate
14	0.50	
15	0.53	
16	0.41	
17	0.52	0.65 Sodium Diethyldithiocarbamate
18	0.51	
19	0.52	0.54 Thiocyanate Acetone System
20	0.24*	
21	0.56	
22	0.49	
23	0.50	0.73 Sodium Diethyldithiocarbamate
24	0.46	
25	0.48	
26	0.53	0.43 A.O.A.C. 2.63 (Starch Iodate)
27	0.46	0.43 Spectrograph (Spark Technique)

Mean: 0.48%
Mean deviation: 0.036%
High result: 0.56%
Low result: 0.41%

Range: 0.15%
High deviation from mean: 0.08%
Low deviation from mean: 0.07%

* Not considered in the average.

Most collaborators submitted results of several determinations. These were averaged to get one value for each participant. Some of the chemists obtained exact results for several determinations while others were observed to have a spread of 0.15 per cent Cu between high and low result. Results for several determinations by other methods also showed quite a spread.

The results of Chemists No. 7 and No. 20 were not considered in the average because of the low percentages obtained. No. 20 did not have the recommended filter available and used a substitute which could account for his low results. Chemist No. 7 analyzed the sample by the sodium diethyldithiocarbamate method for comparative information and his results by this method are very near the average of results of the method under study. Chemist No. 23 also made a sodium diethyldithiocarbamate determination and his results by this method are considerably higher than the average obtained by the method under study. Trials by alternate methods are shown in the table.

METHOD FOR COPPER DETERMINATION IN MINERAL FEEDS

REAGENTS

Ammonium hydroxide.—NH_4OH—C.P.
Copper sulfate.—$CuSO_4 \cdot 5H_2O$—C.P.

STANDARDS

Dissolve 1.9645 g pure copper sulfate in H_2O and dil. to 500 ml. (Each ml of soln is equivalent to 1 mg copper.) Use from one to ten ml of this soln in making up set of standards. Prep. standards in 100 ml pyrex glass-stoppered volumetric flasks. Add 4 ml of concd HCl. Make to volume of 50 ml with H_2O. Warm on water bath at 50°C. Cautiously make to vol. with concd NH_4OH. Stopper and mix thoroly. Make a blank, using all reagents except copper.

DETERMINATION

Ash 2 g sample 2 hours at 600°C., transfer to 200 ml volumetric flask with 20 ml HCl and 50 ml H_2O. Boil 5 min. Make to vol., mix thoroly. Allow soln to settle. Aliquots may be taken from this soln for the detn. of Ca, P, Co, and Cu. Pipet a 50 ml aliquot into a 100 ml glass-stoppered volumetric flask. Warm on water bath at 50°C. Cautiously make to vol. with concd NH_4OH. Mix thoroly. Soln may be filtered, or allowed to settle before comparing in colorimeter or spectrophotometer with standards.

Compare color with standard copper solns in colorimeter, using a red or No. 66 filter. If using a spectrophotometer, a wave length of 620 mμ is recommended.

Report per cent of copper to second place to right of decimal.

DISCUSSION

The method is one of the oldest colorimetric determinations for copper. The acid and hydroxide volumes must be carefully controlled, and the lack of sensitivity of the method is apparent. Other elements are known to interfere in the determination, yet results for this particular sample are fairly good. Cobalt was added to known amounts of copper in the Florida State Laboratory and no interference was noted even when the amount of cobalt was twice the amount normally found in mineral feeds. Some of the collaborators ran into trouble with interferences when the method was tried on other mineral mixtures.

The method was proposed for study with the optimistic hope that a rapid, simple method for copper determinations might be obtained. A

method is needed that will be adaptable to control work on a mass production basis and still be within the limits of an acceptable tolerance for accuracy. This method seems to fulfill this need on this particular sample. There was objection to the sensitivity. The accuracy would be poor on mixtures containing very low amounts of copper. It could be used for the higher levels of copper if interferences could be eliminated; however, we need a method that will cover the entire field. The basic, simple approach is desirable and worthy of further study. Another amine, tetraethylenepentamine, is recommended to be 3.5 times as sensitive as ammonia for copper. The volume of this amine does not have to be carefully controlled, and if interference can be overcome this amine might be successfully substituted for ammonia in the proposed method.

It is recommended* that the study of copper determination in mineral feeds be continued along this line.

The splendid cooperation of all collaborators and their comments have been very helpful and are greatly appreciated.

COLLABORATORS

The order of listing of collaborators has no bearing on the order of listing of results.

W. S. Thompson, Ohio Department of Agriculture Control Laboratory, Columbus, Ohio.

L. A. Koehler, State Laboratories Department, Bismarck, North Dakota.

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* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 48 (1953).

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Wm. J. Ingram, Department of Agriculture, Salem, Oregon.

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Lloyd G. Keirstead and W. T. Mathis, Agricultural Experiment Station, New Haven, Connecticut.

REPORT ON FAT IN FISH MEAL

By M. E. Stansby (Fishery Technological Laboratory, Fish and Wildlife Service, Seattle, Wash.), *Associate Referee*

Work was continued on the determination of fat in fish meal in an effort to obtain a simpler, more rapid, and more accurate method. The acetone extraction method as approved, first action, in *Methods of Analysis*, 7th Ed. (1950), is somewhat more complicated than the simple ether extraction procedure formerly used for fish meal. There have been a number of complaints from industry laboratories that the added step involving acid hydrolysis of the extracted meal followed by a second extraction is too time consuming for routine application.

Several suggestions have been received that perhaps the relatively simple and rapid acid hydrolysis method employing the Mojonnier extraction tube, as now official for fat in fish flesh, might replace the acetone extraction procedure for fish meal. Accordingly, a series of pilchard meals were extracted by this method and by the acetone extraction procedure. A third method was also tried in which the acetone-extracted meal was subjected to the Mojonnier acid hydrolysis procedure in place of the usual hydrochloric acid-Soxhlet extraction. As shown in Table 1, the straight Mojonnier-acid hydrolysis technique invariably gave lower results than the acetone method. When the Mojonnier technic was combined with acetone extraction, variable results were obtained which were sometimes slightly higher and at other times lower than by the regular procedure. In any case, the Mojonnier technic showed no advantage over the acetone extraction procedure, and consequently further attempts to adapt this technique were abandoned.

TABLE 1.—*Oil content of pilchard meals as determined by acetone extraction and Mojonnier-acid digestion methods*

METHOD	OIL CONTENT				
	SAMPLE GC-205	SAMPLE GC-207	SAMPLE GC-213	SAMPLE GC-217	SAMPLE GC-221
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
A.O.A.C. acetone extraction method with HCl digestion and second solvent extraction	11.0	6.9	7.8	9.2	9.3
Mojonnier-acid digestion	9.1	5.9	7.4	7.3	7.9
A.O.A.C. acetone method but substituting Mojonnier-acid hydrolysis for HCl digestion	10.8	7.3	8.0	8.5	9.4

In experiments described in the last report (1), a method was under investigation in which the fish meal was refluxed rather than extracted by the solvent. The advantage is that the characteristics (such as *p*H, water content) of the solvent which comes in contact with the meal could readily be altered. Experiments were reported showing that addition of hydrochloric acid to the acetone solvent resulted in a greater efficiency of extraction of the fat but also resulted in extraction of extraneous material which had to be subsequently removed. These studies have now been extended to determine the effect of adding other substances to the acetone. In all these studies, a technique was adopted that permitted increasing the number of variables which could be investigated in a given time, although it placed the results on an entirely empirical basis. In these tests, the meals were extracted by refluxing with the various solvents for one hour for each stage of the experiment. In some experiments only one stage was involved (total refluxing time then was one hour). On other experiments there were up to four stages (total refluxing time, 4 hours). This gave values only; in most cases all the oil was not extracted from the meal. Thus, while any one series of experiments (reported in any one table) are strictly comparable within that experiment, it is not possible to make direct comparisons of the data in different experiments (reported in the different tables).

In Table 2 are shown results of addition of hydrochloric acid or of ammonium hydroxide to acetone upon gross and ethyl ether purified extracts of fish meal as compared to such extracts employing pure acetone. Addition of either hydrochloric acid or ammonium hydroxide to the acetone resulted in greater total ethyl ether purified extractives than did addition of acetone alone. The acid-acetone mixture gave the highest results. On the other hand, use of ammonium hydroxide gave more nearly

TABLE 2.—*Effect of addition of acid or alkali to acetone in extraction of oil from fish meal*

EXTRACTIVE	OIL CONTENT		
	ACETONE	ACETONE CONTAINING 0.01 N HCl	ACETONE CONTAINING 0.01 N NH_4OH
	per cent	*per cent*	*per cent*
Gross acetone extract	13.39	19.84	14.35
Purified ethyl ether	13.30	15.60	14.09

similar results for the gross extractives (14.35 per cent) and ethyl ether purified extractives (14.09 per cent). This closer agreement is of importance in the development of a rapid method, since it might result in the elimination of a time-consuming ethyl ether purification step.

In Table 3 are shown the effects of addition of acids of three different strengths (hydrochloric, formic, and acetic) to acetone upon the efficiency of extraction of acetone and ethyl ether-soluble materials. The addition of hydrochloric and formic acids yields about the same amount of ethyl ether-soluble extractives. While addition of acetic acid gave lower total recovery of ethyl ether-soluble material than did either of the other acids, most all (98 per cent) of the gross extractives were soluble in ethyl ether, whereas the presence of the other acids in the acetone resulted in considerable extraneous extraction.

It was believed that the amount of water in the acetone might be an important factor in determining the efficiency of extraction of the solvent when a refluxing technique was employed. Preliminary experiments (see Table 4) in which extraction was carried out in two stages, first by refluxing with 100 per cent acetone, then with 75 per cent acetone—25 per cent water, and second when the order of these solvents was reversed, showed that it was very important to remove most of the oil in the initial extraction with 100 per cent acetone before making extractions with acetone containing much water. The reason for this is that when much

TABLE 3.—*Effect of acids of different strengths in acetone upon quantity of extractives obtained from fish meal*

EXTRACTIVES	ACETONE CONTAINING NO ADDED ACID	ACETONE CONTAINING 0.01 N HYDROCHLORIC ACID	ACETONE CONTAINING 0.01 N FORMIC ACID	ACETONE CONTAINING 0.01 N ACETIC ACID
	per cent	*per cent*	*per cent*	*per cent*
A Gross extract	13.0	15.9	15.8	13.5
B Ethyl ether purified extract	12.8	14.3	14.4	13.3

TABLE 4.—*Effect of order of extracting fish meal with acetone and acetone-water mixtures*

STAGE OF EXTRACTION	ACETONE	WATER	OIL CONTENT	
			GROSS EXTRACT	ETHYL ETHER PURIFIED EXTRACT
	per cent	*per cent*	*per cent*	*per cent*
1st Stage	100	0	12.34	12.25
2nd Stage	75	25	6.40	4.84
Total			18.74	17.09
1st Stage	75	25	10.14	6.00
2nd Stage	100	0	7.65	7.56
Total			17.79	13.56

water is present in the acetone its removal results in bumping, spattering, and loss of oil. By removing most of the oil with pure acetone, the subsequent extraction with an acetone-water mixture leaves only a small

TABLE 5.—*Effect of moisture content of acetone used in extracting fish meals upon efficiency of extraction*

STAGE OF EXTRACTION	WATER IN ACETONE	FORMIC ACID IN SOLVENT	OIL CONTENT	
			GROSS EXTRACT	ETHYL EXTRACT PURIFIED EXTRACT
	per cent	*per cent*	*per cent*	*per cent*
1st Stage	0	0	11.82	11.82
2nd Stage	25	0	6.18	3.03
3rd Stage	0	0	0.74	0.64
4th Stage	0	1	0.41	0.21
Total			19.15	15.71
1st Stage	0	0	11.88	11.84
2nd Stage	50	0	8.10	1.79
3rd Stage	0	0	1.39	1.23
4th Stage	0	1	0.42	0.28
Total			21.79	15.14
1st Stage	0	0	11.84	11.88
2nd Stage	75	0	10.45	0.20
3rd Stage	0	0	1.58	1.43
4th Stage	0	1	0.40	0.32
Total			24.30	13.83

amount of oil to be extracted, and any spattering losses are of reduced importance. Accordingly, in all subsequent experiments the meal was given a preliminary refluxing with 100 per cent acetone before applying a solvent containing added water.

In Table 5 is shown the effect of incorporating 25 per cent, 50 per cent, and 75 per cent water in the acetone solvent. Maximum recovery of ethyl ether-soluble material is obtained with the solvent containing 25 per cent water, and this solvent also extracts a minimum of extraneous substances. In this experiment a final extraction with acetone containing acid but no water was also carried out. Addition of acid at this stage caused extraction of only a very small additional quantity of oil, probably no more than would have been extracted by the acetone in the absence of acid. It is concluded that when a water-acetone refluxing solvent is employed, addition of acid is probably unnecessary.

TABLE 6.—*Effect of acetone and acetone-water or acetone-water-acid solvents on efficiency of extraction of fish meal*

STAGE OF EXTRACTION	WATER IN ACETONE	FORMIC ACID SOLUTION IN ACETONE	OIL CONTENT	
			GROSS EXTRACT	ETHYL ETHER PURIFIED EXTRACT
	per cent	*per cent*	*per cent*	*per cent*
1st Stage	0	0	11.90	11.91
2nd Stage	25	0	5.89	3.71
3rd Stage	0	1	1.08	0.60
Total			18.87	16.22
1st Stage	0	0	11.97	11.96
2nd Stage	24	1	6.59	3.13
3rd Stage	0	1	1.01	0.50
Total			19.57	15.59
1st Stage	0	1	15.58	13.81
2nd Stage	24	1	5.68	1.85
3rd Stage	0	1	0.71	0.33
Total			21.97	15.99

This conclusion is further verified by data in Table 6 which again shows that maximum recovery is obtained when acid is absent from the 25 per cent initial acetone solvents. Although a small additional quantity of extractives was obtained in this series when a final extraction was made with an acetone solution containing 1 per cent formic acid but no other water, it is probable that an equal amount of extractives would have been obtained without acid.

Table 7.—*Effect of acetone-water ratio on efficiency of extraction of fish meal*

Stage of extraction	Acetone	Water	Oil content	
			Gross extract	Ethyl ether purified extract
	per cent	*per cent*	*per cent*	*per cent*
1st Stage	100	0	11.80	11.73
2nd Stage	100	0	1.14	1.05
3rd Stage	100	0	0.47	0.19
Total			13.41	12.97
1st Stage	100	0	12.10	12.14
2nd Stage	98	2	2.84	2.67
3rd Stage	100	0	0.41	0.30
Total			15.35	15.11
1st Stage	100	0	11.84	11.85
2nd Stage	96	4	3.35	2.97
3rd Stage	100	0	0.35	0.22
Total			15.54	15.04
1st Stage	100	0	12.18	12.13
2nd Stage	92	8	4.00	3.41
3rd Stage	100	0	0.33	0.20
Total			16.51	15.74
1st Stage	100	0	11.97	11.82
2nd Stage	85	15	5.07	3.64
3rd Stage	100	0	0.39	0.28
Total			17.43	15.74
1st Stage	100	0	11.80	11.65
2nd Stage	75	25	6.30	4.61
3rd Stage	100	0	0.59	0.44
Total			18.69	16.70

In Table 7 are shown results when the quantity of water in the acetone was varied from 0 per cent to 25 per cent. In this series the meal was in each case given an initial and a final extraction with acetone containing no water. The second or middle extraction was made with acetone containing variable amounts of water. Maximum ether soluble extract was obtained when the acetone contained 25 per cent water but the gross extractives in this case contained considerable extraneous (ethyl ether in-

soluble) material. When 8 per cent water was present in the acetone the amount of ethyl ether soluble extractives was quite close to that of the gross extractives and nearly as high as when 25 per cent water was present. Actually, the quantity of gross extractives obtained with 8 per cent water in the acetone was nearly identical (16.5 per cent compared to 16.7 per cent) with the purified extractives obtained when 25 per cent water was present in the acetone. This suggests the possibility of development of a rapid, simple method in which the meal is first refluxed with 100 per cent acetone, then with 92 per cent acetone–8 per cent water.

Studies are continuing to compare results obtained by the use of water in the acetone during refluxing, with results obtained by the regular acetone procedure. Different meals will be tested to see whether such a procedure will give consistent results.

REFERENCES

(1) STANSBY, M. E., *This Journal*, **34**, 549–554 (1951).

REPORT ON CRUDE FAT IN BAKED DOG FOOD

By HAROLD H. HOFFMAN (Florida Department of Agriculture, Tallahassee, Fla.), *Associate Referee*

Budde* has suggested that baked dog foods should be analyzed for fat by a combined acid hydrolysis-ether extraction. Values reported by this procedure approached the theoretical fat content, while acid hydrolysis gave high and ether extraction gave low results.

A collaborative study of this problem has supported Budde's findings, although the variation among laboratories is somewhat greater than desirable for the combination method.

PROPOSED METHOD

Proceed as directed under **13.19** through: "Evap. ethers slowly on steam bath," then continue as follows: Redissolve the fat residue in 20 ml of ethyl ether. Filter thru a small fat-free filter paper into a 50–100 ml beaker that has been previously dried at 100°, cooled in air, and weighed against a counterpoise similarly treated. Using two 10 ml portions ether, rinse original vessel and transfer to filter paper. Evap. ether slowly on steam bath; then dry the fat in a drying oven at 100° to constant wt (ca 90 min.), cool in air, and weigh against counterpoise as before.

COLLABORATIVE WORK

Three baked dog foods and their respective premixes were obtained from different manufacturers, and replicate ground portions were sent to 14 collaborators. Each was requested to determine moisture by **22.3** or **22.7** and crude fat by **22.25** on all samples. On the three baked samples

* Budde, E. F., *This Journal*, **35**, 799 (1952). Grateful acknowledgment is made to this author and to The Quaker Oats Research Laboratories for obtaining and distributing the samples.

additional determinations for fat by acid hydrolysis (**13.19**) were requested, followed by ethyl ether extraction as described above.

Table 1 shows the average moisture contents reported by each collaborator. These were used in converting the averages of the fat values to a dry matter basis as shown in Table 2. This permits comparison of fat in a premix with that in the corresponding baked sample.

Collaborator 12 reported fat determination in duplicate; all others were in triplicate.

TABLE 1.—*Per cent moisture*

COLLABORATOR	SAMPLE NO. 1		SAMPLE NO. 2		SAMPLE NO. 3	
	PREMIX	BAKED	PREMIX	BAKED	PREMIX	BAKED
1	8.94	2.74	8.98	1.49	9.96	4.05
2	8.85	2.50	8.77	1.76	10.57	3.90
3	9.19	2.77	9.22	1.69	11.08	4.19
4	6.02	0.34	5.97	1.27	7.09	3.67
5	8.99	2.95	9.00	1.56	10.78	4.09
6	8.97	2.33	9.03	1.40	11.27	3.83
7	8.30	2.73	9.03	1.43	8.97	3.77
8	9.47	2.97	9.63	1.97	11.27	4.33
9	8.99	2.78	8.92	1.80	10.77	4.35
10	8.75	2.48	8.63	1.49	10.88	3.88
11	8.75	2.44	9.03	1.20	9.92	4.37
12	8.84	2.58	8.90	1.53	10.36	4.20
13	7.23	2.30	7.17	0.98	8.16	3.20

DISCUSSION

Assuming the fat found in the premixes to be the true fat in the corresponding baked samples, **22.25** permits only about 55 per cent recovery after baking, while the proposed modification of **13.19** gives about 112 per cent recovery. This latter recovery, as well as the coefficient of variation, would be more favorable if ıesults for Collaborator 5 were not considered.

Collaborator 4 obtained "much more constant and reproducible values if the first drying and weighing in **13.19** were omitted and only the drying was done as stated in the modification." By this method he reported fat values for samples 1, 2 and 3 of 3.86, 3.56, and 6.26 per cent respectively (when the averages were converted to a dry matter basis). It should be noted that the proposed procedure will not require the drying and weighing to which Collaborator 4 referred. It was only requested in this study to permit comparison of acid hydrolysis with other methods.

Collaborator 9 did not report by **13.19** because the ether solution contained some fine dark particles. He encountered difficulty breaking the emulsions formed during the shaking process and felt they prevented the

TABLE 2.—*Per cent crude fat (dry basis)*

COLLABORATOR	SAMPLE NO. 1				SAMPLE NO. 2				SAMPLE NO. 3			
	PREMIX	BAKED			PREMIX	BAKED			PREMIX	BAKED		
	22.25	22.25	13.19	13.19 (MOD.)	22.25	22.25	13.19	13.19 (MOD.)	22.25	22.25	13.19	13.19 (MOD.)
1	3.63	2.27	4.05	3.28	3.27	1.87	3.85	3.46	6.20	3.45	6.44	5.98
2	3.25	1.99	4.06	4.22	2.89	1.51	4.66	3.89	5.61	3.06	7.27	6.85
3	3.51	1.64	4.23	4.21	3.10	1.55	3.89	3.86	5.95	2.95	6.89	6.85
4	2.79	1.96	4.52	3.41	2.07	1.58	3.18	1.76	5.32	2.83	6.63	4.98
5	3.49	1.79	8.06	7.99	3.31	1.67	7.75	7.69	5.59	3.15	9.24	9.16
6	3.48	1.23	4.17	3.76	3.37	1.59	3.62	3.07	5.97	3.25	7.31	6.27
7	3.41	1.82	4.14	3.63	2.75	1.72	3.78	3.22	5.75	3.33	6.86	6.24
8	3.17	1.93	4.84	3.47	2.66	1.50	4.05	3.03	5.78	3.27	7.66	6.27
9	3.63	2.02	—	3.17	3.04	1.73	—	2.84	5.75	3.20	—	6.43
10	3.50	2.12	4.13	3.37	3.16	1.75	3.45	2.83	5.92	3.29	6.81	6.21
11	3.80	1.95	3.22	2.74	3.65	1.73	3.10	2.99	6.98	3.48	6.26	6.38
12	3.39	1.83	4.06	3.38	3.12	1.57	3.66	2.97	5.75	3.13	6.64	6.05
13*	5.31	—	7.01	6.73	4.76	—	6.23	5.87	7.67	—	9.67	8.82
Average	3.42	1.88	4.41	3.89	3.03	1.65	4.09	3.47	5.88	3.20	7.09	6.47
Standard Deviation	0.26	0.26	1.24	1.36	0.41	0.11	1.29	1.44	0.41	0.19	0.82	0.97
Coefficient of Variation	0.076	0.138	0.281	0.350	0.135	0.067	0.315	0.415	0.070	0.059	0.116	0.150

* Received too late to be included in the average.

solid matter from settling below the outlet of the Röhrig tube in most cases.

RECOMMENDATIONS

It is recommended*—

(1) That the method given herein be made first action.

(2) That collaborative study be undertaken on a greater range of baked dog food samples.

COLLABORATORS

Grateful acknowledgment is made to the following collaborators (not listed in the same order as in the tables):

E. F. Budde, The Quaker Oats Research Laboratories, Chicago, Illinois.

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REPORT ON MICROSCOPY OF FEEDING STUFFS

By J. A. Shrader (Kentucky Agricultural Experiment Station, University of Kentucky, Lexington, Ky.), *Associate Referee*

Recent check samples sent to the various control laboratories for microscopic examination have shown a great lack of uniformity in results. Apparently there are wide differences in the approach to feed microscopy. It is therefore proposed to bring together the experience of several laboratories in this field, with the hope that something may be done in the way of developing uniform methods.

It is recommended† that microscopists from several states and from industry assemble at some central point where facilities for such a meeting are available, such as the University of Kentucky, and carefully go over the various phases of this work. These technicians will be expected to

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 48 (1953).
† For report of Subcommittee A and action of the Association, see *This Journal*, 36, 48 (1953).

contribute of their experience on the various subjects taken up. They will be asked to bring the equipment they use to indicate the various types of apparatus in use and provide the tools for laboratory work during the meeting. In addition, manufacturers of equipment will be invited to bring instruments they have available and, if possible, send representatives to demonstrate and explain them. Where such instruments cannot be loaned for the meeting, descriptive literature could be substituted.

The following subjects have been tentatively outlined as topics to be considered:

1. Apparatus and equipment; types and sizes of hand lenses, low power microscopes, high power microscopes, and possibilities of using projection and the electronic microscope as a means of studying feed samples; types of object illumination including mazda lights, fluorescent lights, ultraviolet and black light; and miscellaneous equipment used by the microscopist, including such items as watch glasses, tweezers, and solutions. Another phase of this general topic would include the types of stages available, whether they are fixed, movable, translucent, etc.

2. Identification techniques, including rudimentary description of cellular structure of both plant and animal material and identification of crystalline chemicals by optical means. This would cover the classification and identification of cellular and crystalline structures by types.

3. Standard comparative samples of ground and unground ingredients, weed seeds, screenings, and other materials such as minerals.

4. Problems in ingredient identification, covering such specific questions as are developed beforehand and at the meeting by those participating.

5. Problems in detecting feed adulteration and substitution of ingredients. Here again a series of problems raised by those participating would be the basis of the discussion.

6. Quick tests of use to the microscopist, including checks for urea, arsenic, fluorine, copper, manganese, iron, salt, etc.

7. Ingredient estimation, methods, accuracy, and uses.

8. A discussion of definitions in the handbook of the Association of American Feed Control Officials and their relationship to the work of microscopy.

9. Interpretation of results of microscopy to manufacturers, dealers, and the public, together with methods of reporting to these groups, use in publications, educational uses, etc.

10. Literature on microscopy.

11. Photo-microscopy, including the discussion and demonstration of equipment, cost of operation, etc. This would include both black and white and color.

12. Consideration of program for developing needed materials, and perhaps indicating some research necessary for promoting microscopy as an instrument of feed control.

This meeting would be confined to three days, and as now planned will be held at Lexington, Kentucky, early in 1953. If a more suitable place and time can be suggested by those interested, such will of course be considered. Those expecting to participate should contact the Department of Feed and Fertilizer, Kentucky Agricultural Experiment Station, Lexington, Kentucky.

REPORT ON MILK BY-PRODUCTS IN MIXED FEEDS

TOTAL SOLIDS DETERMINATION

By Ara O. Call (Western Condensing Company, Appleton, Wis.), *Associate Referee*

Because of the rather widespread interest in the determination of total solids in condensed milk by-product feeds, this problem was selected for collaborative study this year.

It has been generally recognized that in the various drying methods for determining total solids, volatile substances other than water may be driven off. In the case of milk products without developed acidity, the amounts of such substances may be insignificant from a practical point of view. However, in the case of most condensed milk by-product feeds there has been appreciable acid development, and conventional oven drying moisture methods drive off significant amounts of volatile acids. It seems that in the interest of honesty and fair dealing, such non-water volatile materials should be included as part of the total solids, for it is recognized that they also have nutritive value. A method for remedying this difficulty was suggested to this Association as long ago as the October 1934 Annual Meeting. In a note, J. W. E. Harrisson (1) recommended the addition of zinc oxide to the sample to bind the acids and thus prevent their loss in the drying operation. He also pointed out that charring of the dried sample would be reduced with this modification of the official method. American Butter Institute, in its Laboratory Manual (2), pages 53–54, gives a method, "The Determination of Total Solids in Condensed Buttermilk," which employs the addition of zinc oxide to the sample. It is believed that this practice had its origin following Mr. Harrisson's note. At the 1946 meeting, the Associate Referee, R. E. Bergman, gave a report on sampling and analysis of condensed buttermilk (3). It was shown at that time that significantly higher total solids values would be obtained when the volatile acids were neutralized with zinc oxide and retained in the solids determination. At the 1951 meeting, Call and Van Poucke, in a contributed paper (4), pointed out that neutralization of high acid samples would result in higher solids values. It was urged at that time that a collaborative study on the determination of solids in condensed milk by-product feeds be undertaken.

The Associate Referee contacted many individuals and laboratories

TABLE 1.—*Composition of samples*

	SAMPLE 1		SAMPLE 2		SAMPLE 3	
	FOUND	GUARANTEED	FOUND	GUARANTEED	FOUND	GUARANTEED
Per cent total solids	58.4	62.0	55.0	55.0	41.2*	36.0
Per cent acidity (as lactic acid)	5.1	—	5.8	—	5.0	4.0‡
Per cent lactose monohydrate	37.2	42.0	32.0	27.5	10.2	18.0†
Per cent protein ($N \times 6.25$)	7.9	9.0	9.3	9.0	8.4	10.0
Per cent ash	7.2	8.5	7.8	—	4.1	3.5
Per cent fat	—	0.5	—	0.5	—	2.0
Per cent fiber	—	None	—	None	—	None
*p*H (1 to 1)	4.0	—	3.9	—	3.7	—
Riboflavin, mg/lb	5.0	5.49	13.0	11.5	5.5	4.5

* Some of the ground grain was screened from this sample before submitting it to collaborators. This may explain the difference in solids values.
† Guarantee is for Nitrogen Free Extract rather than lactose.
‡ Guarantee is for lactic acid, rather than acidity (as lactic acid).

asking for their cooperation in the collaborative study. Sixteen individuals volunteered their services. Three samples of commercial condensed milk by-products originating from different manufacturers were submitted for the study. Table 1 shows the guaranteed analyses and the results obtained in the Associate Referee's laboratory. Collaborators were asked to make total solids determinations by three different methods, and where possible to report their results in triplicate.

Description of Methods. Method A was a copy of **15.14** (5) with the suggestion that approximately 1 gram samples be used and sufficient distilled water be added to distribute the sample evenly on the bottom of the dish. This method specifies heating on a steam bath followed by three hours in an air oven at 98 to 100°C.

Method B was the same as Method A except an excess of zinc oxide was to be added to each sample and the calculation modified to take into account the water formed by neutralizing the acid, which would be lost in the drying. A sample calculation was furnished.

Method C was the same as Method A except that the sample was to be neutralized by adding standard NaOH solution. A correction was to be made for the added sodium. A sample calculation was also furnished.

A summary of the mean values reported by each collaborator is given in Table 2. It is apparent that Method A gives significantly lower values than obtained by Methods B and C. This is to be expected because of the neutralization of volatile acids in Methods B and C. The salts of these acids are not volatile and hence are retained upon heating. It will be noted that Method A values are only 94 to 98.7 per cent as great as those obtained with Methods B and C.

TABLE 2.—*Total solids in condensed milk by-products feeds*
Average values reported by collaborators

COLLABORATOR	SAMPLE 1			SAMPLE 2			SAMPLE 3		
	METHOD A	B	C	METHOD A	B	C	METHOD A	B	C
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cebt*	*per cent*	*per cent*
1	56.79	59.82	60.58	55.57	51.48	50.69	31.83	35.54	34.78
2	60.34	60.07	61.05	55.62	56.33	57.39	34.87	35.81	35.78
3	58.93	60.32	59.74	54.88	56.24	57.05	34.12	36.17	36.02
4	58.14	60.62	59.97	54.19	57.34	55.64	34.28	36.71	35.59
5	60.37	61.05	60.80	56.74	57.20	57.47	34.93	36.40	36.81
6	57.94	60.02	60.60	54.64	56.79	56.70	33.54	36.17	35.48
7	58.79	60.96	59.51	55.62	57.33	56.24	34.20	36.21	35.45
8	59.72	60.80	60.40	55.75	56.88	57.56	34.00	36.06	34.85
9	60.47	61.25	61.27	56.32	59.89	57.61	34.33	38.73	36.25
10	60.84	60.80	62.10	57.23	57.03	57.61	35.46	36.47	35.89
11	57.97	56.92	57.17	53.85	55.11	55.02	33.34	34.76	34.97
12	60.85	59.52	63.26	56.45	56.77	58.59	34.40	36.00	36.17
13	58.13	59.28	58.72	59.79	56.19	55.82	33.14	34.94	34.88
14	58.16	—	61.85	55.31	—	58.33	33.93	—	37.02
15	58.09	59.90	58.98	54.86	56.49	55.93	33.31	35.85	34.87
16	59.32	60.25	60.19	55.45	56.49	56.54	34.11	36.26	35.71
Mean	59.05	60.11	60.39	55.77	56.50	56.51	33.99	36.14	35.66
		Sample 1			*Sample 2*			*Sample 3*	
$\frac{A}{B} \times 100$		98.24			98.71			94.05	
$\frac{A}{C} \times 100$		97.78			98.69			95.32	

Table 3 shows the precision achieved by each collaborator. It is interesting to note more than a seven-fold difference between collaborators in their ability to replicate their own results.

Statistical data given in Table 4 shows there is no significant difference in the precision (not accuracy) of Methods A, B, or C in the hands of different collaborators. An analyst is equally as liable to report abnormally high or abnormally low values with any of the three methods. Because differences between collaborators appear to be the major source of variation, it is not possible to distinguish differences between methods. Table 4 also shows there is no significant difference between the precision achieved in measuring the total solids in the various samples. The total solids level, in the range studied, seems not to influence the precision of measurement.

COMMENTS OF COLLABORATORS

No. 6. "By physical appearance Method A shows much caramelization with probable lactone formation, etc. If a direct heating method is to be used, a vacuum drying method such as *Methods of Analysis*, 7th Ed., **22.3** or **15.81** should be investigated, or even drying at 70° in vacuum." This collaborator also pointed out that,

TABLE 3.—*Evaluation of precision among triplicate results of individual collaborators.*—All methods, all products

COLLABORATOR†	ESTIMATED STANDARD DEVIATION
	per cent
8	0.151
16*	0.188
7	0.197
5	0.204
4	0.260
10*	0.296
6	0.296
15	0.314
3*	0.486
9	0.517
13*	0.550
2	0.728
1	1.054
11	1.167

† Collaborators 12 and 14 not included because 12 had only duplicates, and 14 omitted Method B.
* 8 sets of triplicates rather than 9 as for others.

with Method A, continued heating after the prescribed 3 hours showed additional losses in weight. This was not the case with Methods B and C.

No. 7. "The dried residue of Method A was much darker than that obtained by Methods B or C; however, C was slightly darker than B. Of the two neutralization modifications, I prefer the sodium hydroxide neutralization or Method C."

No. 8. "I have tried every method I could think of to study these results and see if there were any conclusions that might be drawn. The only conclusion I can draw at this time is that the Method A, which is the A.O.A.C. method at the present time, gives the most consistent results. It also gives the lowest results."

No. 11. "Methods A and C had considerable caramelization while Method B

TABLE 4.—*Estimated standard derivations by method and sample*

Based on the sums of squared deviations from the group mean, with 13 to 15 collaborators reporting triplicate results

	SAMPLE 1	SAMPLE 2	SAMPLE 3	MEAN COEF. OF VARIATION
	per cent	*per cent*	*per cent*	*per cent*
Method A	1.334	1.125	0.904	2.318
Method B	1.122	1.857	0.930	2.567
Method C	1.319*	1.000*	0.700*	1.976*
Mean Coef. of Variation	2.107	2.365	2.398	

* Collaborator 1 results not included.

showed only slight caramelization. Drying time on steam-bath varied from 20 minutes to 90 minutes."

No. 15. "(1) Methods A and C, a 'caramelizing' or charring of samples seems to occur during drying, which did not appear in Method B. (2) Some difficulty in determining end point in neutralization in Method C; also in determination of acidity."

Collaborators were also asked to make total solids determinations by any other methods they thought were applicable. Four of them submitted results and six different methods or modifications were represented. A tabulation of these data follows (Table 5):

TABLE 5.—*Per cent total solids obtained by other methods*

METHOD	MOJONNIER			TOLUENE DIST. (22.5)		KARL FISCHER	60–70° FOR 15 HRS.	OVER H_2SO_4 (22.6)	METHOD C EXCEPT NEUT. WITH $Ca(OH)_2$	AVERAGE
COLLABORATOR NUMBER	4*	7	8	1	8	7	8	7	8	
Sample 1	59.55	60.08	61.46	58.5	61.0	58.4	61.38	61.91	60.30	60.29
Sample 2	55.40	56.50	56.64	54.0	—	55.9	56.65	57.69	56.93	56.21
Sample 3	34.45	35.44	35.47	33.0	—	35.0	35.25	37.62	36.03	35.28

* Average of 3 analysts.

The Mojonnier method for total solids is one of long standing in the dairy industry and it is widely used. It is very rapid, but requires rather elaborate special equipment. The Toluene Distillation Method for Moisture (**22.5**) is an official method; however, it is not well suited to the measurement of total solids in condensed, relatively high moisture products. The Karl Fischer Method for Moisture employs a chemical measurement of water. It is very useful in certain cases, but it too does not lend itself to the measurement of total solids in such products as those in question. The collaborator using it pointed out that poor replications were experienced due to difficulty in putting the samples into solution. Prolonged drying at reduced temperatures as well as drying without heat over sulfuric acid (**22.6**) both have limited practical application because of the time required. Collaborator 8 made a series of tests using a standard $Ca(OH)_2$ solution to neutralize the acidity instead of NaOH as specified in Method C. The results obtained are quite comparable to other neutralization methods, i.e., Methods B and C.

It will be noticed that in every case the average total solids for each sample by all of these methods is higher than that obtained by Method A, but compares favorably with values obtained by Methods B and C (compare Table 2).

SUMMARY

Three commercial samples of condensed milk by-product feeds were submitted to 16 collaborators for total solids determinations in triplicate by the following three different methods:

A. (AOAC **15.14**) A 10–15 minute preliminary heating on a steam bath followed by three hours in an air oven at 98–100°. The residue is reported as total solids.

B. The same procedure as A, except an excess of zinc oxide is added to the dish before adding the sample, to neutralize any acids present.

C. Similar to A except the sample is neutralized with a measured amount of standard NaOH solution prior to drying. Appropriate corrections are made in the calculations in both Methods B and C.

The results obtained by Method A were significantly lower than those obtained by either Methods B or C, being only 94 to 98.7 per cent as great. There was no difference in the replicability of any of the methods, but the ability of some collaborators to repeat themselves varied rather widely.

Four of the collaborators made total solids determinations by six other methods or modifications, and although the results varied somewhat, the average value for each sample was much nearer to the values obtained by Methods B and C than those obtained by A, the official method.

RECOMMENDATIONS

The Associate Referee recommends* (1) that collaborative studies on the determination of total solids in condensed milk by-product feeds be continued and (2) that consideration be given to the adoption of a total solids method wherein the acidity of the sample is neutralized prior to oven drying.

COLLABORATORS

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* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 48 (1953).

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W. S. Thompson, Ohio Dept. of Agriculture, Section of Feeds & Fertilizers, Columbus, Ohio.

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(5) *Methods of Analysis*, A.O.A.C., 7th Ed. (1950), p. 231.

REPORT ON DRUGS IN FEEDS

DETERMINATION OF NITROPHENIDE AND ENHEPTIN ®

By Richard T. Merwin (Connecticut Agricultural Experiment Station, New Haven, Conn.), *Associate Referee*

Methods of assay published by Lederle Laboratories for its products, nitrophenide (1) and Enheptin ® (2), used for the control of coccidiosis in chickens and blackhead in turkeys, respectively, were studied collaboratively this year. An evaluation of both methods is presented in this report.

In their main essentials, the methods are those studied a year ago, with minor modifications. Lederle Method 1806 replaces Method 1785. The latter had been used by feed control laboratories until November 15, 1951, when it was superseded by Method 1806. The method for Enheptin, as originally published, was studied last year and was again included in this year's collaboration, with a slight alteration in extraction procedure.

Both methods are colorimetric. Nitrophenide contains two nitro groups (m-m′-dinitrodiphenyldisulfide) that are reduced to form amino groups for diazotization and coupling with N′-(1-naphthyl)-ethylenediamine dihydrochloride. The purplish-red complex is read spectrophotometrically at 545 mμ. Enheptin (2-amino-5-nitrothiazole) develops a yellow color in alkaline solution. When the drug is reduced with sodium hydrosulfite it loses its characteristic color and the difference in density readings be-

tween the reduced and unreduced compound is used as a basis for measurement of concentration.

Recent trends in animal feed medication greatly complicate drug assays of products containing mixtures of growth-promoting and disease-inhibiting substances. More and more, organic arsenicals are being added to feeds as growth stimulators, largely to give the effect of better growth by producing better coloring and improved feathering. Such an arsenical as *p*-arsanilic acid incorporated in feeds with nitrophenide, marketed as Megasul A, 25 per cent nitrophenide, 12½ per cent arsanilic acid, practically voids the use of Method 1806 for nitrophenide. To find out what effect the arsenical has on regular nitrophenide assays, collaborators were asked to report results on a feed of this type, but only in percentage terms of nitrophenide.

p-Arsanilic acid has an amino group in the *para* position that readily diazotizes and couples with N′-(1-napthyl)-ethylenediamine dihydrochloride. The color complex shows maximum absorption at 538 mμ and this peak is too close to the 545 mμ absorbancy of nitrophenide to avoid interference. Corrections for the additive densities of the two, by reading unreduced drug solutions as blanks and calculating by differences, give low recoveries for nitrophenide. Specifically, corrections for density due to *p*-arsanilic acid result in lower densities to be assigned for evaluation of nitrophenide.

Three samples containing: (1) 0.0125% nitrophenide; (2) 0.0125% nitrophenide and 0.0062% *p*-arsanilic acid; and (3) 0.100% Enheptin, were sent to each collaborator. Vials of the recrystallized drugs to be used as standards were enclosed in each sample bottle. Each collaborator received identical samples from the same gross preparations that had been mixed thoroughly with known quantities of recrystallized drug. No procedural instructions or cautions as to technique were issued other than those appearing in copies of the methods.

COLLABORATIVE METHOD FOR NITROPHENIDE

REAGENTS

(a) $Na_2S_2O_4$.

(b) 0.5% $NaNO_2$ solution (freshly prepared).

(c) 2.5% solution of ammonium sulfamate.

(d) 0.1% solution of N′-(1-naphthyl)-ethylenediamine dihydrochloride. (Store in dark bottle).

(e) Phosphate buffer *p*H 6.6—To 1 liter flask add 13.62 g monopotassium phosphate (KH_2PO_4), dissolve in H_2O, add 35.6 ml of 1 *N* NaOH, make to vol., and mix.

DETERMINATION

To each of two 250 ml Erlenmeyer flasks add 2 g of ground sample and 50 ml of the phosphate buffer soln. To the second flask add 1 g $Na_2S_2O_4$. Place both flasks in boiling water bath for 20 min., remove, and while hot add 10 ml of concd HCl; then immediately aerate both flasks with a stream of air for 40 min.

Transfer solns to 100 ml flasks, make to vol., mix and allow to stand one-half hour. Filter through Whatman No. 42 paper, discarding first 10 ml of filtrate.

To 4 ml aliquots in small beakers, add 1 ml of the $NaNO_2$ soln to the reduced and unreduced samples. After 5 min. add 1 ml of the $NH_4SO_3NH_2$ soln and after two min., 1 ml of the N'-(1-naphthyl)-ethylenediamine dihydrochloride soln. Add 10 ml of water to make vol. of 17 ml and read absorbancy at 545 mμ on spectrophotometer. Subtract absorbancy of unreduced soln from reduced soln and refer to standard curve for concn.

Prepare standard soln by dissolving 0.0125 g of recrystallized m-m'-dinitrodiphenyldisulfide in acetone and making to vol. of 250 ml with acetone. Transfer aliquots of 2.5, 5.0, 7.5, and 10 ml. to 250 ml Erlenmeyer flasks and evap., using gentle stream of air. To each flask and to a blank flask, add 1 g of $Na_2S_2O_4$ and proceed as in assay. Plot absorbancy on graph paper for resulting readings at 5, 10, 15, and 20 mmg concns.

COLLABORATIVE METHOD FOR ENHEPTIN®

REAGENTS

(a) Acetone.

(b) 5% solution of NH_4Cl in H_2O.

(c) Boric acid buffer *p*H 9.0 prepared by making two solutions: **A**—6.203 g boric acid and 7.456 g KCl made to 500 ml with H_2O; and **B**—0.2 *M* soln of sodium hydroxide. Take 50 ml of Soln A and 21.40 ml of Soln B and make to 200 ml with H_2O. Make 1% soln of sodium hydrosulfite in *p*H 9.0 boric acid buffer and use not later than 10 minutes after prepn.

DETERMINATION

Weigh 2 g of ground feed into a 50 ml volumetric flask, add 10 ml of acetone and stand two min., swirling occasionally. Make to vol. with water, mix, and filter quickly through coarse paper. Transfer 25 ml aliquot to 50 ml flask, add 15 ml of the NH_4Cl soln and mix. Make to vol. with water and filter through Whatman No. 42 paper, discarding first 10 ml of filtrate.

Add a 5 ml aliquot to each of two small beakers. To the first add 0.5 ml of the 1% sodium hydrosulfite soln in boric acid buffer. Make both volumes to 10 ml and read on a spectrophotometer against H_2O at 388.5 mμ. Subtract the reading of the reduced soln from that of the unreduced and compare resulting absorbancy to standard curve.

Prepare standard soln by dissolving 100 mg of recrystallized 2-amino-5-nitrothiazole in 100 ml of acetone and make to vol. of 1 l with H_2O. Transfer aliquots of 4, 8, 12, 16, and 20 ml to 100 ml volumetric flasks and dil. to vol. with water. Treat 5 ml aliquots of each diln as in assay procedure, reading the absorbancy of the unreduced soln against the reduced soln as a blank, obtaining readings at 20, 40, 60, 80, and 100 mmg concns.

Results of the collaborative study of the methods appear in Tables 1 and 2.

Table 1.—*Analyses of feeds containing nitrophenide*

Collaborator	Nitrophenide, per cent			In presence of 0.0062% p-arsanilic acid		
	Present	Found	Averages	Present	Found	Averages
No. 1	0.0125	0.0137		0.0125	0.0094	
		0.0137			0.0094	
		0.0131			0.0091	
		0.0131	0.0134		0.0088	0.0092
No. 2		0.0100			0.0110	
		0.0100			0.0100	
		0.0100	0.0100		0.0100	0.0103
No. 3		0.0109			0.0090	
		0.0099			0.0070	
		0.0100			0.0060	
		0.0109	0.0104		0.0050	0.0068
No. 4		0.0100			0.0053	
		0.0084			0.0053	
		0.0112			0.0055	
		0.0087	0.0096		0.0056	0.0054
No. 5		0.0103			0.0084	
		0.0105			0.0085	
		0.0106			0.0087	
		0.0105	0.0105		0.0088	0.0086
No. 6		0.0103			0.0065	
		0.0097			0.0073	
		0.0095			0.0060	
		0.0097			0.0073	
		0.0097	0.0098		0.0056	0.0065
No. 7		0.0120				
		0.0121				
		0.0123				
		0.0128				
		0.0121				
		0.0122				
		0.0125				
		0.0128	0.0124			
No. 8		0.0183	0.0183		0.0211	0.0211
No. 9		0.0110			0.0096	
		0.0110			0.0086	
		0.0112			0.0085	
		0.0110	0.0111		0.0085	0.0088
Average of all, omitting No. 8			0.0109			0.0079
Per cent recovery, omitting No. 8			87.2			63.2

TABLE 2.—*Analyses of feeds containing Enheptin*

COLLABORATOR	ENHEPTIN ®, PER CENT		
	PRESENT	FOUND	AVERAGES
No. 1	0.100	0.086 0.085 0.081 0.081	0.083
No. 2		0.098 0.092 0.093 0.095	0.095
No. 3		0.079 0.078 0.077 0.079	0.078
No. 4		0.068 0.066 0.066 0.068	0.067
No. 5		0.096 0.090 0.092 0.093	0.093
No. 6		0.096 0.096 0.090 0.090	0.093
No. 8		0.036	0.036
No. 9		0.097 0.096 0.096 0.096	0.096
Average of all Per cent recovery			0.086 86.0

COMMENTS AND DISCUSSION

There was a surprising lack of favorable or unfavorable comment from collaborators. One remarked that the aeration process in the nitrophenide method "is a messy procedure that always worries me." Those familiar with the method readily admit that the process is disagreeable and lengthens the time of assay considerably. There is always the possibility that all the sulfur will not be precipitated and subsequent filtration, even after a half-hour wait, may result in turbid filtrates.

Among the nine collaborators, only one reported reasonably theoretical recovery; one other was fairly close with an average of plus 7 per cent. One, who submitted only one figure, which presumably represents his average of several determinations, was 46 per cent above the known amount of drug. The six remaining collaborators were in fair agreement among themselves, but their average is only 81.8 per cent of the theoretical figure.

It will be noted that in general the six reporting the lowest figures show good individual reproducibility. If their results on all feeds should prove to be consistently low, individual factors depending on the analysts' experience might possibly be applied as corrections to be used in control work. However, such a method of assay would always be empirical and inexact.

The published method for nitrophenide does include provision for use of a correction factor for low level feeds. It is recommended that the adjusted absorbency be divided by 0.97 before comparison with the standard curve. Apparently a small amount of unreduced nitrophenide is absorbed by the feed. Collaborators were not asked to use such a correction because such a small factor would not be of aid in evaluating the method itself.

The Associate Referee feels there is a strong possibility that closer theoretical recovery of nitrophenide will be attained only after the drug is freed from the feed before it is reduced. Clear, protein-free solutions for reduction seem essential if better results are to be obtained. To some extent, this conclusion is substantiated by the hundreds of extractions and reductions tried experimentally in the author's laboratory. But a suitable method of extraction remains the basic problem.

In Table 1 it will be seen that recoveries were quite low for nitrophenide on Sample No. 2. The effect of *p*-arsanilic acid on nitrophenide determinations makes the present method impracticable in application to mixtures of the two drugs.

Results on the Enheptin assays were better than those for nitrophenide. Four laboratories reported figures within reasonable range of 0.100%. Two collaborators did not submit figures. Three others probably encountered difficulties in extraction. The low results indicate a smaller difference in densities between the reduced and unreduced solutions.

The comment of one collaborator is pertinent. He found during the Enheptin assays that the use of wide-mouthed 50 ml volumetric flasks was superior to the use of narrow-mouthed flasks. Feed tended to clog the stems of the latter during filtration. The Associate Referee has always used the wide-mouthed flask, from which the feed solutions may be poured onto the filter quickly and without difficulty. Clogging, by preventing rapid filtration, might cause some absorption of the dissolved drug.

The extraction technique may not have been clear as it appeared in the method. When water is added to the acetone-feed solution of the drug,

there is formation of foam. For this reason, a wide-mouthed flask is essential in making to volume accurately. As a matter of necessary technique, making to mark, mixing, and filtering should be performed as quickly as possible. Letting the water-acetone solution of unfiltered feed stand too long may result in some absorption of enheptin. Unfortunately, this point was not stressed sufficiently.

Accordingly, the method for Enheptin should be altered to read:

"Weigh two g of ground feed into a wide-mouthed 50 ml volumetric flask, add 10 ml of acetone and let stand two minutes, swirling occasionally. Make to vol. with H_2O, mix thoroughly, and *immediately* filter rapidly through coarse paper."

One final matter of Enheptin assay technique should be emphasized. The solution of sodium hydrosulfite in boric acid buffer must be used not later than 10 minutes after preparation. Although this precautionary statement appears under the list of reagents in the method, it should be re-emphasized in the body of the method, as follows: "To the first, add 0.5 ml of the freshly prepared 1% solution of sodium hydrosulfite in boric acid buffer." This solution becomes turbid on standing, and if used in such a condition causes higher density readings on reduced solutions.

CONCLUSIONS

The wide range in results of the collaborators' reports on the nitrophenide method indicates that further study of the method, or development of a more accurate method, is necessary. Lack of sufficient stress on critical technique in the Enheptin method as written probably accounts for the lower figures obtained by three of the seven reporting collaborators. As soon as these points of technique are clearly understood and applied, consistently accurate results should be obtained.

ACKNOWLEDGMENT

Sincere appreciation for their very helpful cooperation is expressed to the following collaborators:

Sherman R. Squires, Assistant Chemist, The Connecticut Agricultural Experiment Station, New Haven, Conn.; M. P. Etheredge, State Chemist, Mississippi State Chemical Laboratory, State College, Miss.; Sigmund W. Senn, Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.; Van P. Entwhistle, Supervising Feed Chemist, and Chester A. Luhman, Senior Feed Chemist, Feed Laboratory, Department of Agriculture, Sacramento, Calif.; James N. Turner, Chief Chemist, The Park and Pollard Co., Buffalo, N. Y.; W. R. Flach, Laboratory Director, Eastern States Farmers' Exchange, Buffalo, N. Y.; John Reid, Chemist, Wirthmore Research Laboratory, Malden, Mass.; Charles E. Weber, Chemist, New Jersey Agricultural Experiment Station, New Brunswick, N. J., and Roland W. Gilbert, Assistant Research Professor in Agricultural

Chemistry, University of Rhode Island Agricultural Experiment Station, Kingston, R. I.

RECOMMENDATIONS

In the report of Subcommittee A on Recommendations of Referees, *This Journal*, **35**, 43 (1952), omission appears of a recommendation for the status of the sulfaquinoxaline method. The Associate Referee had recommended continuation of studies of the method (adopted first action) with a view to shortening the method. Such studies have subsequently proved such a condensation impracticable. The Associate Referee, therefore, recommends,*

(1) That the method for sulfaquinoxaline be made official.

(2) That the method for Enheptin ® with alterations as outlined, be resubmitted for collaborative study.

(3) That collaborative studies of a method for nitrophenide be continued.

REFERENCES

(1) "Analyses of Various Concentrations of Nitrophenide Premixes and Finished Feeds," Methods of Assay No. 1806, Animal Feed Department, Lederle Laboratories Division, American Cyanamid Company, 30 Rockefeller Plaza, New York 20, N. Y.

(2) "Method of Assaying Feeds for Enheptin," Animal Feed Department, Lederle Laboratories Division, American Cyanamid Company.

No report was given on tankage (hide, hoof, horn, and hair content); or on crude protein in feeding stuffs.

The contributed paper, "Determination of Nitrofurazone in Feeds," by V. R. Ells, E. S. McKay, and H. E. Paul, appears on page 415.

REPORT ON SOILS AND LIMING MATERIALS

By W. H. MacIntire (The University of Tennessee Agricultural Experiment Station, Knoxville 16, Tenn.), *Referee*

The contribution from the Referee has been solely of an advisory character. The work done by the Associate Referees is reported so far as their reports have been received. Upon the basis of their work and recommendations, the following recommendations† are made:

(1) That studies on the "combination dithizone-spectrographic method" and on the polarographic procedure for the determination of zinc in soils be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 48 (1953).
† For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 51 (1953).

(2) That the study of the determination of copper in soils be continued.

(3) That the utilization of carmin as an indicator in the determination of the boron content of soils be studied further, and that *p*-nitro benzenazo-1,8-dihydroxynaphthalene-3,6-disulfonic acid, or "chromotrope-B," be studied as a suitable reagent in that determination.

(4) That the neutral calcium acetate method for the replacement and the determination of exchangeable hydrogen of soils be adopted as official.

(5) That for the calculation of the desired degree of base saturation, use should be made of the analytical values for exchangeable hydrogen and of total exchangeable metal cation content of the soil; and for the determination of practical "lime requirements" of soils below that of *p*H 7, the amount should be calculated upon the basis of the degree of base saturation desired. Official, first action.

(6) That the survey and comparison of methods for the determination of phosphorus, (a) that fraction in "available" state and (b) the proportion of organic-inorganic forms therein (*This Journal*, **30**, 43 (1947)), be continued.

(7) That the survey and comparison of methods for the determination of exchangeable potassium in soils (*This Journal*, **30**, 44 (1947)) be continued, and that a detailed procedure be prepared and studied collaboratively.

(8) That the Associate Refereeship on exchangeable calcium and magnesium be continued.

(9) That the double distillation method for fluorine (*This Journal*, **34**, 58 (1951)), as reworded by the Associate Referee, be made official.

REPORT ON POTASSIUM ANALYSES IN SOILS AND PLANT MATERIALS BY FLAME PHOTOMETER METHODS

By A. Mehlich, *Associate Referee*, and M. E. Harward (North Carolina Agricultural Experiment Station, University of North Carolina, Raleigh, N. C.)

Previous reports* have shown that the flame photometer technique is suitable for the determination of exchangeable potassium. Conventional extraction or leaching methods in which the soil-solution ratios were 1:10 or greater were found to result in efficient replacement of K. Substantial errors were encountered, however, because of faulty calibration, instrument and analyst errors, and variable extraction procedures. In order to eliminate errors, due to extraction procedures, it was recommended that a detailed outline of a standard procedure be prepared and submitted to

* *This Journal*, **34**, 589 (1951); **35**, 588 (1952).

the collaborators for their suggestions. Such a procedure has been prepared and made available for study. Meanwhile, it has been found desirable to continue the study on the causes of error due to instrument and analyst variation. The present report deals only with the results obtained with a Perkin-Elmer Model 52A flame photometer in studies involving, 1) varying concentrations of Li as the internal standard, 2) day to day variations, and 3) analyst variations.

Effect of Li and K concentrations on reproducibility of flame photometer measurements of potassium. The effect of Li concentration at varying concentrations of K on the reproducibility of flame photometric measurements is shown in Table 1. The results indicate that the percentage error

TABLE 1.—*Effect of Li and K concentration on reproducibility of Perkin-Elmer flame photometer measurements of K*

K	Li CONCENTRATIONS—P.P.M.						
	50	100	150	200	250	50	100
	ANALYST 1					ANALYST 2	
p.p.m.	*Percentage deviation*					*Percentage deviation*	
9	5.4	5.4	1.3	2.1	1.3	38.7	12.5
35	0.6	2.4	0.8	2.0	2.8	6.9	3.5
59	2.0	0.9	0.5	0.7	2.0	5.6	1.5
82	2.9	1.4	0.8	1.2	0.3	3.1	1.2
105	2.2	1.4	1.9	0.9	0.5	2.6	0.7
Average	2.6	2.1	1.1	1.4	1.4	11.4	3.9

* Calculations are based on the averages of three independent measurements.

is greatest at the lower concentrations of K as well as of Li. The sensitivity of the instrument was low at the 25 and 50 p.p.m. Li level. The measurements, therefore, required meticulous care by Analyst 1. Analyst 2 was instructed to make some of these measurements with the speed and care used in routine analysis. These results, for the 50 and 100 p.p.m. Li concentrations, are also given in Table 1. These data show clearly a very much greater error with 50 than with 100 p.p.m. Li. Apparently it is essential that the optimum concentration of Li must be determined for each instrument.

Reproducibility of flame photometer measurements of potash from plant materials. The reproducibility of flame measurements also has been studied on the ashings of plant materials. Determinations with 100 p.p.m. Li as the internal standards were made on two days by two analysts. The results presented in Table 2 show the mean percentage difference from 7 ashings to range from 0.6 to 3.4 as day to day variation, and to be 1.7 and 2.6 for Analysts 1 and 2 respectively. However, these results

TABLE 2.—*Reproducibility of Perkin-Elmer flame photometer measurements of potash in plant materials*

PLANT MATERIAL	ANALYST	DAY	REPLICATE ASHING OF SAME MATERIAL								MEAN PERCENTAGE DIFFERENCE
			1	2	3	4	5	6	7	AV.	
			K_2O in plant material—per cent								
Corn Leaves	1	1	3.80	3.61	3.58	3.63	3.53	3.63	3.63	3.63	
		2	3.81	3.60	3.66	3.63	3.56	3.61	3.63	3.64	
		Difference	0.01	0.01	0.08	0.0	0.03	0.02	0.0	0.02	0.6
	2	1	3.79	3.55	3.67	3.67	3.55	3.60	3.61	3.63	
		2	3.84	3.60	3.66	3.69	3.64	3.75	3.63	3.69	
		Difference	0.05	0.05	0.01	0.02	0.09	0.15	0.02	0.06	1.6
Lespedeza	1	1	1.21	1.13	1.08	1.17	1.14	1.17	1.20	1.16	
		2	1.23	1.19	1.19	1.16	1.19	1.19	1.21	1.19	
		Difference	0.02	0.06	0.08	0.01	0.05	0.02	0.01	0.04	3.4
	2	1	1.20	1.22	1.18	1.15	1.19	1.18	1.19	1.19	
		2	1.25	1.17	1.15	1.24	1.21	1.24	1.18	1.21	
		Difference	0.05	0.05	0.03	0.09	0.02	0.06	0.01	0.04	3.4
Orchard Grass	1	1	3.19	3.13	3.13	3.08	3.13	3.08	3.18	3.13	
		2	3.19	3.19	3.07	3.13	3.00	3.13	3.21	3.13	
		Difference	0.0	0.06	0.06	0.05	0.13	0.05	0.03	0.05	1.6
	2	1	3.28	3.20	3.17	3.14	3.03	3.03	3.23	3.15	
		2	3.19	3.15	3.08	3.19	3.13	3.24	3.21	3.17	
		Difference	0.09	0.05	0.09	0.05	0.10	0.21	0.02	0.09	2.9
Peanut Tops	1	1	1.40	1.49	1.40	1.45	1.45	1.49	1.45	1.45	
		2	1.43	1.44	1.40	1.41	1.45	1.49	1.45	1.44	
		Difference	0.03	0.05	0.0	0.04	0.0	0.0	0.0	0.02	1.4
	2	1	1.51	1.46	1.40	1.42	1.40	1.42	1.50	1.44	
		2	1.53	1.45	1.43	1.50	1.48	1.48	1.50	1.48	
		Difference	0.02	0.01	0.03	0.08	0.08	0.06	0.0	0.04	2.7
Fescue	1	1	2.51	2.56	2.47	2.47	2.47	2.51	2.54	2.50	
		2	2.50	2.58	2.37	2.42	2.41	2.50	2.48	2.47	
		Difference	0.01	0.02	0.10	0.05	0.06	0.01	0.06	0.04	1.6
	2	1	2.50	2.50	2.46	2.45	2.40	2.40	2.52	2.46	
		2	2.47	2.61	2.41	2.52	2.54	2.45	2.51	2.50	
		Difference	0.03	0.11	0.05	0.07	0.14	0.05	0.01	0.07	2.8
Cotton Stems	1	1	1.86	1.90	1.90	1.90	1.86	1.86	1.93	1.89	
		2	1.88	1.88	1.86	1.87	1.83	1.91	1.88	1.87	
		Difference	0.02	0.02	0.04	0.03	0.03	0.05	0.05	0.03	1.6
	2	1	1.88	1.95	1.88	1.85	1.90	1.88	1.99	1.90	
		2	1.85	1.95	1.94	1.94	1.94	1.92	1.97	1.93	
		Difference	0.03	0.0	0.06	0.09	0.04	0.04	0.02	0.04	2.1

Mean percentage—between days

Analyst 1	1.7
Analyst 2	2.6

show that the flame photometer technique is suitable for the determination of potassium in plant materials.

This study suggests that instrument performance is largely dependent on the care of the analysts and on the selection of an optimum concentration of Li as the internal standard.

REPORT ON EXCHANGEABLE HYDROGEN IN SOILS

THE TITRATION OF EXCHANGEABLE HYDROGEN WITH CALCIUM CARBONATE AND RESULTANT *p*H VALUES OF CERTAIN SOILS

BY W. M. SHAW (The University of Tennessee Agricultural Experiment Station, Knoxville 16, Tenn.), *Associate Referee*

In accord with the recommendations of the Associate Referee in his 1951 report (6), the calcium acetate procedure for the determination of the exchangeable hydrogen content of soils was subjected to further study. The immediate objectives were: first, to test the reliance of the calcium acetate exchangeable hydrogen method in predicting the $CaCO_3$ required to produce a near-neutral reaction when applied to a larger number of soils than was previously reported; second, to expedite the reaction between $CaCO_3$ and soils so that it may serve as a convenient check on the effectiveness of the predicted $CaCO_3$ requirement by that method.

EXPEDITION OF THE REACTION BETWEEN CALCIUM CARBONATE AND SOILS

In the previously reported studies the reaction of $CaCO_3$ with soils was carried out in moist contact at the temperature of 30°C. A period of four weeks was required for the complete reaction of the 325-mesh calcite when applied in quantities equivalent to the exchangeable hydrogen content. The influence of biologically engendered nitrate and sulfate upon soil *p*H was noted. In the present study, the reaction was carried out during a six-hour period over the steam bath (about 95°C.) and the completeness of the reaction was tested through the analysis of residual carbonate on fifteen soils, as given in Table 1.

Two chemical properties of each soil, the metal cation content and exchangeable hydrogen values, had to be determined as requisite information, and the computed $CaCO_3$ additions, given in Table 1, were based on these. The cation exchange capacities and degrees of base saturation were computed from those two values. The metal cation content was determined by the extraction of 20-gram charges of air-dry soil with a neutral normal solution of ammonium acetate and the evaporation and ignition of the residue at 500°C. The residue was dissolved in an excess of 0.2 *N* HCl at room temperature. The filtrate was boiled, cooled, and back-titrated with standard 0.1 *N* NaOH to the clear yellow color of methyl red indicator. The exchangeable hydrogen was determined by the Ca-

acetate procedure (5, 6). The cation exchange capacities were taken as the sums of the metal cations and the exchangeable hydrogen values.

To test the completeness of the $CaCO_3$-soil reaction, the carbonate additions were made on 10-gram soil charges in the form of 325-mesh marble, in quantities to supply percentages of cation-exchange capacities of 80, 90, 100, 110, 120 per cent, inclusive of the metal cation content of each soil. The $CaCO_3$ additions of 10 and 20 per cent above and below the 100 per cent cation saturations were made in anticipation of probable plus and minus deviations in the determined exchangeable hydrogen values as compared with $CaCO_3$-soil reaction to *p*H 7 of some of the soils.

The soil carbonate mixtures were placed in the 125 ml fat-extraction flasks, wetted with 10 ml of H_2O, stirred, and washed down with about ten additional ml of water. The flasks were placed over 250 ml beakers on a hot plate about half full of boiling water and evaporated to dryness. The wetting and evaporation were repeated three times. The complete process requires from five to six hours on the water bath. The residual carbonate was determined directly by connecting the carbonate-reaction flask to the apparatus for CO_2 determination by the method of steam distillation (4).

The results of residual carbonate determinations, given in the last section of Table 1, prove that the described treatment of the soil-$CaCO_3$ systems offers a method for complete decomposition of the additive $CaCO_3$ when the additions are within 100, 110, and in many instances even up to the 120 per cent levels of cation exchange capacities of the soils. The significance of this finding is that it points to the possibility of a laboratory procedure for *p*H-titration curves on acidic soils to *p*H 7 through reactions with solid $CaCO_3$, and without the necessity of analyzing for or accounting for residual carbonate. This conclusion is supported by the unanimity of the carbonate analysis results with fifteen distinct soil types and is re-enforced by the fact that these were derived from a diversity of adsorption complexes, extending from nearly 100 per cent organic (the Portsmouth muck) to 100 per cent mineral (the Cumberland and Susquehanna clay subsoils). Among the soils of Table 1 are soils from a number of states other than Tennessee. Portsmouth muck is from Florida; Sassafrass sandy loam from Mississippi; Talladega clay loam from North Carolina; Volusia silt loam from New York; Wooster silt loam from Ohio, and Susquehanna clay subsoil from Alabama. Thus titrations of the soil acidity by means of solid $CaCO_3$ can be carried out with ease and expedition, and the results are more reliable than can be obtained with the customary titrations with $Ca(OH)_2$ or $Ba(OH)_2$.

GRADUAL NEUTRALIZATION OF SOILS BY MEANS OF CALCIUM CARBONATE AND RESULTANT *p*H VALUES

Thirteen of the most acidic soils listed in Table 1 were selected for establishing a more extensive series of neutralization degrees in relation to

resultant *p*H values. The $CaCO_3$ additions were made in increments of 20 per cent of the respective cation exchange capacities, except that increments near the 100 per cent value were made in 10 per cent intervals. Since there was no necessity for determining residual carbonate, the procedure of the soil-$CaCO_3$ reaction was simplified further by placing the soil-carbonate mixtures in 25 ml Coors evaporating dishes, wetting to a thin paste, and stirring with a small glass rod, which was left in the dish. The evaporations were accomplished by setting the dishes over 150 ml beakers kept on a hot plate, two-thirds filled with gently boiling water. As soon as they were dry, the soils were wetted again by means of a stream of hot water, stirred gently, and again evaporated to dryness. The wetting and drying was continued for seven or eight cycles. (It was found most convenient to prepare the soil-carbonate mixtures and to wet them the afternoon prior to the day of the evaporation. In the case of highly organic soils, such as Portsmouth muck, this step is essential to avoid the rapid generation of CO_2, which would cause overflow of the wetted mixture.) After the last drying, the dishes were removed from the steam bath and cooled to room temperature. Their contents were made to pastes with distilled water, stirred several times during thirty minutes, and *p*H values were determined in the same containers by means of the glass electrode. The *p*H results on the thirteen soils at various degrees of base saturation are given in Table 2.

The point of greatest interest in Table 2 are the *p*H values in the 100 per cent base saturation column. It bears on the main question as to whether the $CaCO_3$ additions, based on exchangeable hydrogen determinations by the Ca-acetate procedure, would, after complete reaction with the soil, impart to such a soil a *p*H of 7 or near that value. The results of Table 2 show that in nine of the thirteen tests, *p*H values were $7.0 \pm .1$; *p*H values of three others were between 6.6 and 6.7; only the Portsmouth muck gave the low *p*H of 6.2. The next point of importance relates to the *p*H values at 80 per cent base saturation; it has been indicated (1) that this percentage of base saturation presents the ideal condition for general crop production. The *p*H values of the 80 per cent base saturation column generally range from 6.4 to 6.6. The exceptions to this rule are the Portsmouth muck with *p*H 5.8; the Hartsells with *p*H 6.2; the Cumberland subsoil with 5.8; and the Susquehanna subsoil with 5.9. In the case of the two soils first mentioned, it is possible that the Ca-acetate procedure gave a somewhat low estimate of the $CaCO_3$ requirement of these two highly organic adsorption complexes. In the case of the two subsoils, the deviations in *p*H at 80 per cent are due to the sharp break in the neutralization curves between the 80 and 100 per cent neutralization. On the whole, it may be stated that with nearly all soil samples (twelve out of thirteen instances) subjected to this test, the $CaCO_3$ additions, in accordance with the Ca-acetate indications (and after complete reaction with the soils) induced soil reactions of practical neutrality, or *p*H 6.7 to 7.0.

In view of these results, it is difficult to interpret such a statement as: "At present, the lime requirement of a soil is sometimes based on the amount of exchangeable hydrogen displaced by leaching the soil with a solution of a salt. These procedures for the most part displace only 60 to 75 per cent of the exchangeable hydrogen present" (3, p. 41). If this statement expresses the relative hydrogen displacement of neutral salts, as compared with the soil's capacity for $CaCO_3$ decomposition after a period of a year or longer, the expression is readily understood. In such event, however, the reference point would not be a soil of *p*H 7 any longer, but one possessing a *p*H of 7.5 to 8.2 (2), and one containing more calcium than is usually desirable for practical agriculture.

THE NATURE OF THE *p*H-SOIL NEUTRALIZATION CURVES

Examination of the *p*H changes with increase in degree of base saturation of soils in Table 2 reveals a general tendency towards a 0.4 to 0.5 *p*H increase for each 20 per cent increase in base saturation. Notable exception to this rule is the very slow *p*H rise in the acid range of the Montmorillonitic Susquehanna subsoil, followed by a rapid rise in the region beyond the 80 per cent saturation. In all cases, however, the *p*H-soil titration curves with $CaCO_3$ show perceptible although not well-defined transitions which cause appreciable deviation from a straight-line relationship between *p*H and base saturation degree. Graphical presentation of typical titration curves from Table 2 are given in Figure 1.

It should be recalled that in order to translate any calculated $CaCO_3$ requirement from the *p*H-base saturation curve into pounds of calcium carbonate per acre, it is necessary to have a knowledge of the exchange capacity of the soil or of its metal cation content from which the cation exchange capacity may be obtained by the simple addition of metal cations plus exchangeable hydrogen content. In view of the individual peculiarities of the *p*H titration curves of soils, coupled with appreciable fluctuations in *p*H values of a soil of a given state of saturation, it is believed that a better plan for estimating the lime ($CaCO_3$) requirement of an acid soil would be to base such an estimate on the percentage of base saturation desired, whether 60, 80, or 100 per cent. Data for such estimation can be supplied by two simple laboratory determinations; the exchangeable hydrogen content by the Ca-acetate method (5, 6), and total exchange metal cation content obtained in the above-described manner. The metal content is very useful information and can be used, through addition with exchangeable hydrogen, to give the cation exchange capacity of the soil. For checking on the *p*H value that the calculated liming would induce, it is only necessary to add the requisite quantity of finely divided $CaCO_3$ (325-mesh) to a 10-gram soil sample, subject the mixture to the described wetting and drying on a steam bath, and obtain the *p*H value the same day.

TABLE 1.—*Completeness of reaction of calcium carbonate added to soils in proportions of 80 to 120 per cent of their cation exchange capacities after wetting and drying six hours on steam bath*

SOIL TYPE	EXCHANGE CATION VALUES					ADDITION OF $CaCO_3$ (GRAMS PER 10g*) FOR BASE SATN. PERCENTAGES					UNREACTED $CaCO_3$ RESIDUE—mEq./100g.				
	METAL CATIONS	EXCH. HYDRO.	EXCH. CAPACITY	BASE SATN.	pH	80	90	100	110	120	80	90	100	110	120
	mEq.	*mEq.*	*mEq.*	*per cent*											
Baxter Silt Loam	2.02	4.4	6.42	31	5.4	.0157	.0189	.0220	.0254	.0286	0.0	0.0	0.0	0.1	0.1
Bolton Silt Loam	7.70	4.3	12.00	64	6.2	.0108	.0168	.0215	.0288	.0348	0.0	0.0	0.0	0.0	0.3
Dickson Silt Loam	1.63	4.2	5.83	28	5.0	.0152	.0181	.0210	.0239	.0268	0.0	0.0	0.0	0.0	0.0
Cumberland Clay Subs.	1.10	6.9	8.00	14	5.0	.0264	.0304	.0345	.0384	.0424	0.0	0.0	0.0	0.0	0.0
Hagerston Silt Loam	6.01	2.6	8.61	70	6.1	.0043	.0086	.0130	.0172	.0215	0.0	0.0	0.0	0.3	0.3
Montevallo Silt Loam	2.81	5.1	7.91	36	5.1	.0174	.0214	.0255	.0293	.0332	0.0	0.0	0.0	0.0	0.1
Sequoia Silt Loam	3.70	5.4	9.10	41	5.4	.0177	.0223	.0270	.0314	.0359	0.0	0.0	0.0	0.0	0.0
Tellico Silt Loam	6.40	5.8	12.20	52	5.8	.0171	.0232	.0290	.0354	.0415	0.0	0.0	0.0	0.2	0.3
Portsmouth Muck	2.30	50.0	52.30	4	4.0	.1076	.2236	.2500	.2756	.3016	0.0	0.0	0.2	0.8	1.2
Sassafras Sandy Loam	0.80	3.4	4.20	19	5.2	.0128	.0149	.0170	.0191	.0212	0.0	0.0	0.0	0.0	0.0
Susquehanna Clay Subs.	2.80	27.0	29.80	9	4.0	.1065	.1215	.1350	.1515	.1665	0.0	0.0	0.0	0.5	2.3
Talladega Clay Loam	1.40	8.3	9.70	14	5.0	.0320	.0368	.0415	.0465	.0514	0.0	0.0	0.0	0.0	0 0
Volusia Silt Loam	7.30	6.1	13.40	55	5.7	.0168	.0235	.0305	.0364	.0431	0.0	0.0	0.0	0.0	0.8
Wooster Silt Loam	2.39	6.0	8.39	28	5.0	.0218	.0260	.0300	.0344	.0386	0.0	0.0	0.0	0.0	0.0
Hartsells Sandy Loam	1.20	11.8	13.00	9	4.7	.0461	.0526	.0590	.0656	.0721	0.0	0.0	0.0	0.0	0.0

* The $CaCO_3$ addition in grams per 10 grams of soil multiplied by 200, give mEq. per 100 grams of soil.

TABLE 2.—*Effect of varying degrees of neutralization of exchangeable hydrogen through reaction with calcium carbonate upon pH values of certain soils and subsoils*

SOIL TYPE	CATION EXCHANGE CAPACITY	INITIAL BASE SATURATION DEGREE	$CaCO_3$ REQUIRED (GRAMS PER 10 G OF SOIL)* FOR PERCENTAGES OF BASE SATURATION								pH OF SOILS AT PERCENTAGE OF BASE SATURATION								
			20	40	60	80	90	100	110	120	INITIAL	20	40	60	80	90	100	110	120
	mEq.	*per cent*																	
Portsmouth muck	52.0	4	.0416	.0936	.1456	.1976	.2236	.2496	.2756	.3016	3.8	4.4	4.8	5.2	5.8	6.0	6.2	6.6	6.7
Hartsells sandy loam	13.0	9	.0072	.0202	.0332	.0462	.0526	.0592	.0656	.0722	4.8	5.0	5.4	5.8	6.2	6.4	6.6	6.8	6.9
Susquehanna clay subsoil	30.0	9	.0165	.0465	.0765	.1065	.1215	.1365	.1515	.1665	4.4	4.7	4.9	5.0	5.2	5.8	7.0	7.9	8.1
Talladega clay loam	9.7	14	.0029	.0126	.0223	.0320	.0368	.0417	.0465	.0514	4.9	5.0	5.4	5.9	6.5	6.6	6.7	6.9	7.1
Cumberland clay subsoil	8.0	14	.0024	.0104	.0184	.0264	.0304	.0344	.0384	.0424	4.4	4.4	5.0	5.3	5.9	6.4	6.7	6.9	7.2
Sassafras sandy loam	4.2	19	—	.0044	.0086	.0128	.0149	.0170	.0191	.0212	5.2	—	5.8	6.2	6.6	6.8	7.0	7.2	7.5
Dickson silt loam	5.8	28	—	.0035	.0093	.0151	.0181	.0209	.0239	.0267	5.2	—	5.5	5.7	6.3	6.8	6.9	7.1	7.2
Wooster silt loam	8.4	28	—	.0050	.0134	.0218	.0260	.0302	.0344	.0386	5.2	—	5.2	5.8	6.5	6.8	7.0	7.2	7.3
Baxter silt loam	6.4	31	—	.0028	.0092	.0156	.0189	.0220	.0254	.0284	5.4	—	5.6	6.0	6.5	6.9	7.0	7.1	7.2
Montevallo silt loam	7.9	36	—	.0016	.0095	.0174	.0214	.0253	.0293	.0332	5.1	—	5.3	5.8	6.4	6.7	6.9	7.1	7.3
Sequoia silt loam	9.1	41	—	—	—	.0177	.0223	.0270	.0314	.0359	5.4	—	—	—	6.6	6.8	7.0	7.2	7.4
Tellico sandy loam	12.2	52	—	—	—	.0171	.0232	.0293	.0354	.0415	5.8	—	—	—	6.7	6.9	7.1	7.3	7.5
Volusia silt loam	13.4	55	—	—	—	.0168	.0235	.0302	.0364	.0431	5.7	—	—	—	6.4	6.7	6.9	7.1	7.3

* The grams $CaCO_3$ added per 10 grams of soil times 200 = mEq. per 100 grams.

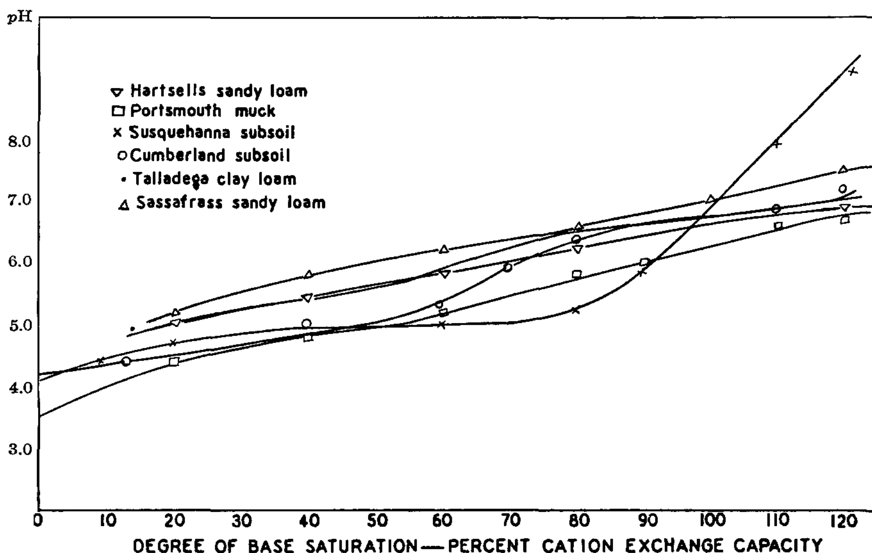

FIG. 1.—*p*H-Degree of Base Saturation Curves of Typical Soils and Subsoils, after Reaction with Calcium Carbonate.

SUMMARY

Fifteen soils and subsoils of diverse types of adsorption complexes were analyzed for metal cation and exchangeable hydrogen content. The acidic soils were neutralized in steps of 20 per cent and up to 120 per cent of their cation exchange capacities, through reaction with $CaCO_3$, by repeated wetting and drying on a steam bath. This ensures complete soil-$CaCO_3$ reaction within six hours contact. In fourteen out of fifteen cases, the $CaCO_3$ additives, as predicted by the Ca-acetate method for exchangeable hydrogen determination, induced soil reactions of practical neutrality, *p*H 6.6 to 7.1.

The *p*H-soil neutralization curves were too irregular to serve as a basis for computing the $CaCO_3$ requirement for *p*H values of soils below that of 7. It is suggested that for soil reactions below *p*H 7, the $CaCO_3$ requirements should be calculated on the basis of percentage base-saturation desired, i.e. 60, 70, or 80 per cent. This can be done readily from two simple analytical determinations, each of which yields useful information as to soil properties. These are the total exchangeable metal cation and exchangeable hydrogen contents.

RECOMMENDATIONS

It is recommended*—

1. That the neutral calcium acetate method for the replacement and

* For report of Subcommittee A and action of the Association, see *This Journal*, 30, 51 (1953).

the determination of exchangeable hydrogen of soils be adopted as official.

2. That for the determination of practical "lime requirements" of soils below that of *p*H 7, the amount should be calculated on the basis of the degree of base saturation desired.

3. That for the calculation of the desired degree of base saturation, use should be made of the analytical values of total exchangeable metal cation and exchangeable hydrogen contents of the soil.

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REPORT ON FLUORINE IN SOILS

A STUDY OF THE TITRATION PHASE OF THE DETERMINATION OF FLUORINE

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In 1951 (3), ten collaborators participated in a study on the determination of the fluorine content of soils by the direct double distillation-thorium nitrate titration method (1, 4). In that study, each collaborator performed his own distillations under stipulated conditions which were designed to assure uniformity. The titration step also was carried out under uniform directions. The results obtained by the various laboratories showed decided lack of agreement. Although some workers expressed complete confidence in the reliability of the thorium nitrate titration, it has been observed that different individuals, or even the same individual, obtain significantly variable results from the titration alone.

The present collaborative study was devised to eliminate as many variables as possible except those of the titration step. To accomplish this, the Associate Referee supplied a quantity of distillate prepared by the double distillation of individual charges of a fluoride-bearing soil. The 250 ml collections were composited, and a portion was sent to each collaborator for *p*H adjustment and titration under conditions as uniform as could be attained by following identical directions.

The following objectives were contemplated:

(1) To ascertain whether the variation in the determined fluorine content of soil was due to the titration step or to the distillation.

Table 1.—*Fluorine content of soil distillate. Results of A.O.A.C. collaborative studies*

Collaborator	Aliquot	pH of buffered solution			Titer 0.01 N $Th(NO_3)_4$			Fluorine: micrograms			Fluorine: p.p.m.			AV
	ml	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*1*	*2*	*3*	*p.p.m.*
1[a]	100	2.60	2.60	2.60	0.87	0.86	0.85	59	58	57	295	290	285	290
	50	2.60	2.60	2.60	0.54	0.56	0.52	29	30	28	290	300	280	290
2[b]	100	3.00	3.02	2.98	0.61	0.60	0.59	60	58.5	57.5	300	292	288	293
	50	3.05	2.98	3.00	0.36	0.37	0.37	31.5	32.2	32.2	315	322	322	320
3[a]	100	3.00	3.00	3.00	0.63	0.62	0.62	63	62	62	315	310	310	312
	50	3.00	3.00	—	0.35	0.35	—	30	30	—	300	300	—	300
4[b]	100	2.97	2.97	2.97	0.62	0.63	0.63	51.5	52.0	52.0	258	260	260	259
	50	2.97	2.97	2.97	0.38	0.36	0.37	27.5	25.5	26.5	275	255	265	265
5[b]	100	2.97	2.97	3.01	0.730	0.746	0.739	58.6	60.2	59.0	293	301	295	296
	50	2.97	2.97	3.01	0.420	0.428	0.437	27.4	28.3	29.7	274	283	297	285
6[b]	100	3.00	3.00	3.00	0.515	0.520	0.520	55.0	55.5	55.5	275.0	277.5	277.5	276.7
	50	3.00	3.00	3.00	0.265	0.265	0.270	27.0	27.0	27.5	270.0	270.0	275.0	271.7
7[a]	100	3.00	3.00	3.00				58	63	60	290	315	300	302
	50	3.00	3.00	3.00				34	34	31	340	340	310	330
8[a]	100	—	3.00	3.00	—	0.67	0.67	—	57	57	—	285	285	285
	50	3.00	3.00	3.00	0.39	0.39	0.38	29	29	28	290	290	280	287
9[b,d]	100	3.10	3.10	3.10	0.52	0.57	0.53	43	(av.)	—	—	—	—	d
	50	3.10	3.10	3.10	0.31	0.30	0.30	13	13	13	—	—	—	d
10[a]	100	3.00	3.01	3.01	0.540	0.535	0.530	56.6	56.1	55.5	283	281	278	281
	50	3.00	3.00	3.00	0.295	0.300	0.300	27.7	28.3	28.3	277	283	283	281
Average	100													289
	50													292
	over-all													290

[a] White base used.
[b] Transparent stand over white base used.
[c] 0.00209 N solution used, delivered from 5 ml. buret graduated in 0.02 ml.
[d] Values of 216 and 130 p.p.m., respectively, for the 100 ml and 50 ml aliquots not included in average.

(2) To compare results obtained by titration of two fluorine levels through use of two different aliquots.
(3) To eliminate as many variables as possible other than personal factors.
(4) To compare possible effects of variation in pH of the buffered solution upon the titration values.
(5) To test the use of a transparent stand over a white base as a possible aid in discerning the end point.

The soil distillate sent to each collaborator for titration was obtained by means of steam-distillation of 0.5 gram charges of a Maury silt loam from sulfuric acid at 165°C. Each 500 ml distillate was evaporated to 10–15 ml, and then distilled from $HClO_4$ at 130°C., to collect 250 ml. The multiple distillates were composited and 500 ml portions of the composite were sent to each collaborator.

Each collaborator was requested to prepare his own buffer solution and 0.01 N thorium nitrate solution, standardized against sodium fluoride solution (not distilled), and then to make triplicate titrations of 100 ml and of 50 ml aliquots of the soil distillate, using Alizarin Red S indicator. A report sheet showing conversion of the titers of the 100 ml and 50 ml aliquots into p.p.m. F in the soil was provided.

DETERMINATION OF FLUORINE BY THORIUM NITRATE TITRATION

REAGENTS

(a) *Buffer solution.*—Dissolve 9.45 g monochloracetic acid in 50 ml distd water. To this soln add a soln contg 2 g NaOH and add H_2O to make 100 ml.

(b) *Alizarin red.*—Dissolve 1 g Alizarin Red S in 1 liter H_2O; dil. 10 ml of this solution with 90 ml H_2O to make final soln. For smaller amounts, use same ratio.

(c) *Thorium nitrate.*—Make 0.01 N soln from dry $Th(NO_3)_4 \cdot 4H_2O$ crystals. Prepare standardization curve by titrating against aliquots of standard sodium fluoride soln, ranging from zero to 100 mmg of F in increments of 10 mmg. 1 ml of 0.01 N thorium nitrate will be equivalent to ca 100 mmg F.

TITRATION

Measure triplicate 100 ml aliquots of the soil distillate into 150 ml beakers. Add 2 ml Alizarin Red S indicator, neutralize the sample by dropwise addn of 0.50 N NaOH until pink color develops. Add 1 ml of buffer, which will bring soln to pH 3.0 $\pm$.1.

Provide a stand made by supporting a sheet of clear glass, or plexiglass, $1\frac{1}{2}$ inches above the white base of a buret stand. Place a fluorescent titration light behind the buret stand in the usual way and, with the beaker placed on the stand in front of the light, titrate to the incipient salmon-pink end point.

In case this stand arrangement is not available, or cannot readily be improvised, use the lighting system customarily used for titration in your laboratory.

Record the buret reading, compute the results from the standard curve, and report the values on the sheet provided.

Repeat the titrations on triplicate 50 ml aliquots diluted to 100 ml and report as for the 100 ml titrations.

COLLABORATORS

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COMMENTS OF COLLABORATORS

The collaborators made several suggestions which should be helpful to those engaged in the titration of fluoride. The comments are quoted as follows:

(1) By our "Direct Titration" method—285 ppm; by our "Colorimetric Titration" method—285 ppm. Titration made on white base.

(2) The .01 *N* solution seems to be too strong for titration of the 50 ml aliquot. When .005 *N* was used, the values obtained averaged 296 p.p.m. Titration was made on transparent stand over white base.

(3) The $Th(NO_3)_4$ is not quite 0.01 *N*. Titration of 50 ml of the distillate by the method currently used in our laboratory, chrome azurol indicator, *p*H 3.3, gave a value of 300 p.p.m. Titration made on white base.

(4) Titration made on transparent stand over white base.

(5) Each operator has prepared his own Th-standardization curve. The two operators collaborated to the point of obtaining agreement within 3% on a laboratory distillate, before the Tennessee distillate was titrated. Each operator then made his individual titrations of the Tennessee distillate.

By the micro method (our usual method of titrating) we obtained 14.7 mmg F per 25 ml or 58.8 mmg per 100 ml. The micro-titration was done *after* the requested titrations had been made and reported. (58.8 mmg is equivalent to 294 p.p.m.) Titration made on transparent stand over white base.

(6) The ml of 0.01 *N* $Th(NO_3)_4$ listed in the above table do not include the amount required for end point with no fluoride present. Titration made on transparent stand over white base.

(7) A weaker solution of $Th(NO_3)_4$ was used (.00209 *N*) with a large buret (5 ml graduated at 0.02 ml). The normality shown (.00209) was not theoretical, but is based on actual fluoride titer. Titration made on white base.

(8) In our customary titration procedure, a 0.05 difference in *p*H makes an appreciable error. We are wondering if the .2 variance allowed in this soil titration might not also be a source of error. At the 90 and 100 microgram levels, the exact end point was hard to find and also deviated from the straight line graph. Titration made on white base.

(9) Use was made of a microburet (5 ml calibrated in 0.01). It seems the

titrating solution is too strong; a more dilute solution should make the end point more easily seen. The end point seems very hard to get under any circumstances. We have used a light showing thru a hole in the titrating table, covered with white glass. Titration made on transparent stand over white base.

(10) A titration blank of .06 ml was subtracted from the above titrations. The fluoride value of the thorium nitrate was equivalent to 0.118 mg F/ml. Titration made on transparent stand over white base.

DISCUSSION OF RESULTS

The results reported by the ten collaborators are shown in Table 1, and include the *p*H of the solution, titer, micrograms of fluorine from the standard curve, and p.p.m. fluorine for both the 100 ml and 50 ml aliquots. The values obtained by one collaborator are shown, but, due to certain difficulties reported with the blank determination, his values are not included in the discussion, averages, or statistical analysis.

The *p*H values of the buffered solutions were in close agreement, usually *p*H 3.0±.05. One collaborator reported *p*H 2.60 in all solutions, but apparently that low *p*H value had no adverse effect on the titrations, since his fluorine values were in close agreement with the average. Although some collaborators have expressed the opinion that a very small deviation from the requisite *p*H 3.0 causes appreciable error in the fluoride titration, the present study indicates that a *p*H approximating 3.0 is easily obtained by the stipulated use of the buffer solution and that deviations from that value as occurred in the present comparisons can be tolerated.

There is no apparent correlation between the fluorine values and the type of light background, as between the white base and the transparent stand over the white base. Some operators, however, have expressed satisfaction with the use of the transparent base.

An analysis of variance of the 52 replications reported by the nine collaborators showed:

(1) There was significant lack of agreement between individual collaborators. The L.S.D. at the 5 per cent level was 13 p.p.m., and 18 p.p.m. at the 1 per cent level. Based on the over-all average of 290 p.p.m., four collaborators were within the 13 p.p.m. limit for both the 100 and 50 ml aliquots, seven were within that limit for the 100 ml aliquot, five for the 50 ml aliquot, and one did not come within this range on either aliquot.

(2) There was significant over-all agreement between values from the 100 and 50 ml aliquots. This was true in spite of exceptions in the case of two collaborators who reported decidedly higher values from the 50 ml aliquot, and one who reported a lower value.

(3) For a given collaborator, there was good agreement between replications.

(4) The interaction (collaborators times aliquots titrated) was not signifi-

cant. There was a consistent trend to higher or lower results from either the 100 or 50 ml aliquot; that is, when an analyst obtained high values from the 100 ml aliquots, his values likewise were usually high for the 50 ml aliquot.

A supplemental study was made in the laboratory of the Associate Referee to ascertain the uniformity of the individual distillations. Triplicate distillations of 250 ml each were made from ten still units, numbered from 1 to 10. The average fluorine contents of those triplicate collections, as determined by titration of 100 ml aliquots were respectively 289, 294, 301, 285, 296, 298, 304, 297, 295, and 290 p.p.m. The average fluorine content of the 30 replicated distillates, titrated in the same way and by the same analyst, was 295 p.p.m. This compares to the over-all average fluorine content of 290 p.p.m. as found by 9 collaborators who titrated triplicate 100 ml and 50 ml aliquots of the composite of those distillates. These results indicate a close agreement between individual distillation units. There was also agreement between replications by the same unit.

SUMMARY AND CONCLUSIONS

Ten collaborators participated in the thorium nitrate titration of 100 ml and 50 ml aliquots of a composite soil distillate prepared by direct double distillation of replicate 0.5 gram charges of a Maury Silt Loam.

Although some workers in the field have expressed confidence in the titration technique and lack of necessity for further development in that step, the present study indicates considerable variation in results, probably due chiefly to the personal factor.

The fluorine content found by all collaborators averaged 289 p.p.m. on the 100 ml aliquot, 292 p.p.m. on the 50 ml aliquot, and the over-all average value was 290 p.p.m. The L.S.D. was 13 p.p.m. at the 5 per cent level and 18 p.p.m. at the 1 per cent level. Although most results, compared to the over-all average, were within these limits, the values found for the 100 ml aliquots varied from 315 to 258 p.p.m. F. The maximum and minimum values found on the 50 ml aliquots were 340 and 255 p.p.m. F respectively.

The *p*H measurements of the buffered solutions indicate that use of the buffer as stipulated is adequate to establish the requisite *p*H of approximately 3.0. Also, comparable results were obtained by use of a fluorescent light over either a white base or with a transparent stand over a white base.

Since the results obtained were generally in agreement, it is concluded that the method for titration of fluoride is satisfactory, but is subject to personal factors that can be overcome only through experience and close attention to details of the procedure.

The personal factor is indicated by the fact that identical titers in some cases gave decidedly different values when computed from the standard-

ization curve of two individuals. This is particularly evident in the results by collaborators 3 and 4 for the 100 ml aliquots and a high color blank by collaborator 4 is the probable cause of the low value which he obtained.

The fluorine contents of both the 100 and 50 ml aliquots are within the range for titration with the 0.01 N thorium nitrate solution. Some analysts, however, have expressed a preference for a weaker solution for titration of the 50 ml aliquots.

ACKNOWLEDGMENTS

The collaborators are commended for their careful work which made this study possible, and for the promptness with which they submitted their reports. Also the cooperation of the staff of the Fluorine laboratory of the Tennessee Experiment Station is appreciated, especially the editorial suggestions of Doctor W. H. MacIntire and the assistance of Doctor S. H. Winterberg in making statistical interpretations.

RECOMMENDATIONS*

Although considerable variations in results by different collaborators are apparent solely in the titration phase of the determination of fluoride in soils, these variations obviously are due to the personal visual factor, and no recommendations for additional collaborative study on the titration step are made at this time. Careful attention to the preparation of the standardization curve, the blank, the use of a titrating solution commensurate with the fluoride in the sample aliquot and experience of the operator seem to be the only assurances of uniform results.

One collaborator has referred to implementation and use of an "automatic" titration device of promising accuracy. When this, or some similar instrument or technique (4), is available at several laboratories, its use and adaptation should be the objective of collaborative study.

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No report was given on hydrogen-ion concentration of soils; boron; zinc and copper; exchangeable calcium and magnesium; phosphorus; or molybdenum.

The two contributed papers, "Reaction between Calcium Carbonate and Soils and Determination of Calcium Sorption Capacities," by W. M. Shaw, and "Rapid Determination of Cation and Anion Exchange Properties of Soils," by A. Mehlich, appear on pages 419 and 443 respectively.

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 51 (1953).

REPORT ON SUGARS AND SUGAR PRODUCTS

By CARL F. SNYDER (National Bureau of Standards, Washington 25, D.C.), *Referee*

The Referee concurs in the recommendations* which the several Referees have presented at this meeting.

There appears in the Seventh Edition of *Official Methods of Analysis*, Section **29.17**, a general procedure to be followed in optical rotation methods applicable to sugars and sugar products. Under **(c)** of this section the conversion factors of the different saccharimeter and polarimeter scales are tabulated. In converting saccharimeter readings with white light and the dichromate filter to their equivalent in circular degrees and sodium light the factor 0.34620 is employed. This value was determined by Bates and Jackson† from their observed rotations of the normal quartz plate (100.00°S). It is applicable to solutions of sucrose and many other sugars whose rotatory dispersion values are approximately that of quartz.

TABLE 1.—*Emission of G.E. sodium lamp relative to filtered tungsten*

WAVE LENGTH mμ	PER CENT EMISSION (WITHOUT FILTER)	TUNGSTEN LAMP WITH POTASSIUM DICHROMATE FILTERS	
		3 PER CENT	6 PER CENT
820	4.0		
803	0.12		
797	0.08		
767	0.5		
750	0.11		
738	0.10		
705	0.06		
615	0.5		
589	100.0	86.0	84.0
568	2.3	1.8	1.8
498	0.6	0.3	0.3
466.5	0.2		
330.2	200.0*	15.0	15.0

* Tungsten gives very little radiation at this wave length.

In arriving at this value, these investigators measured the rotation of the normal quartz plate on the saccharimeter and compared it to the rotation on the circular scale polarimeter illuminated with spectrally purified sodium light (5892.5Å). At the last meeting of the International Commission for Uniform Methods of Sugar Analysis (1949), it was recommended that the rotation of the normal quartz plate be measured for the Osram sodium lamp. This lamp has been widely used in European labora-

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 64 (1953).
† *This Journal*, **4**, 330 (1921).

tories, but in this country the General Electric sodium light is generally employed.

Measurements of the rotation of the normal quartz plate were made by the Referee on a Bates type saccharimeter with white light and dichromate filter, and on the polarimeter illuminated by the G.E. sodium lamp and dichromate filter. Thirty-one series of observations were made at 20°C. on each instrument. The average value obtained for the normal quartz plate, reading 100°S on the saccharimeter, was 34.621 circular degrees on the polarimeter with the G.E. sodium light. This agrees with the value of 34.620 obtained by Bates and Jackson for spectrally purified sodium light.

The G.E. sodium lamp was tested with the Beckman DU spectrophotometer by Allan Gee of the Surface Chemistry Section, National Bureau of Standards. Line emission spectra are given in Table 1. The intensity of these lines are expressed relative to the spectrum of a 6-volt 32 c.p. incandescent tungsten lamp operated by three large lead storage cells. The ratio of the intensity of the sodium D lines relative to the light of the tungsten lamp of the same wavelength has been set at 100. (Ratios falling below 0.05 were not measured.)

REPORT ON MICRO METHODS OF SUGAR ANALYSIS

By Betty K. Goss (National Bureau of Standards, Washington 25, D.C.), *Associate Referee*

As micro methods of sugar analysis probably find their widest application in medical and biochemical laboratories, it is not surprising that most of them are standardized for dextrose only. However, there are many agencies which have need of micro methods for analysis of other sugars. The sugar industry is interested in a micro method for the determination of invert sugar in refined white sugars, while the current interest in dextran necessitates such methods for use in control laboratories where there is a limited amount of available material. For this purpose, some manufacturers use Somogyi's phosphate method, which will be discussed later.

It seems advisable, first of all, to survey the existing methods. In his article *Les Microméthodes de dosage du sucre dans le sang*, Accoyer (1) presents an extensive review of the subject.

Micro methods for dextrose fall into two major catagories: (1) those methods which depend on the reduction of ferricyanide to ferrocyanide and (2) those involving the reduction of cupric sulfate to cuprous oxide. Generally speaking, copper reagents oxidize sugars more selectively than do the ferricyanide reagents, although the ferricyanide reagents have the advantage of not being so easily re-oxidized by air. Additional methods depend on other reagents, such as Sumner's dinitrosalicylic acid (20),

Lewis and Benedict's picric acid (13), and Benham and Despaul's molybdate method (3), but these are not widely used.

The outstanding example of the ferricyanide reduction methods is that of Folin-Malmros (5), based on that of Hagedorn and Jensen (9). (Many modifications of this method are found in the literature, including those of Horvath and Knehr (11), Reinecke (17), Plumel (16), Wolff and de-Lavergne (21), Park and Johnson (15), Kingsley and Reinhold (12), Herbain (10), and Fonty (8).)

Briefly, the Folin-Malmros method is as follows: place 4.0 ml of the sugar solution in a test tube graduated at 25 ml. Add 2 ml of the potassium ferricyanide solution, followed by 1 ml of cyanide-carbonate solution, mix by lateral shaking, and place in a boiling water bath for 8 minutes. Cool, add 5 ml of the ferric iron-gum ghatti solution, and mix. Let stand for a few minutes and then dilute to the mark with distilled water. Mix well, and allow to stand for 10 minutes. Transfer a portion of the colored solution to a colorimeter tube and read in a colorimeter against a blank tube set at 0, within the next 30 minutes. The method is good up to 0.05 mg glucose/ml.

Plumel published two important notes on the method with regard to more concise definition of the necessary conditions. The colloid protector has been the subject of many of the modifications. Horvath and Knehr suggested the use of Duponal ® instead of gum ghatti; Wolff and deLavergne used gelatin and varied the reagents of Folin-Malmros slightly; Park and Johnson also used Duponal in their adaptation of the method to very small quantites of glucose (1–9 mmg glucose/1–3 ml); Herbain adapted the method to small quantities of glucose (5–25 mmg glucose/2 ml) and was able to dispense with the addition of the protective substance; and recently Fonty used PVP (polyvinylpyrrolidone) as the colloid protector. Reinecke also adapted the method to very small quantities of blood.

There are several copper reduction methods of particular interest. These are the Somogyi carbonate method (19), which is found in *Methods of Analysis*, 6th Ed. (1945), the Somogyi phosphate method (19) which will be discussed here, and the Folin and Wu method (7) which was described in the August, 1952, issue of *This Journal*, under the report on micro methods of sugar analysis. In addition, the quantitative method of Benedict (2) should be mentioned.

Somogyi's phosphate modification has been under study because of its current popularity with dextran manufacturers who have adopted it for use in checking dextran samples for molecular weight.

The method has the advantages of ease of operation, speed, stability of reagent, and a relatively uncritical boiling time.

SOMOGYI PHOSPHATE METHOD

REAGENTS

Copper-phosphate solution.—Dissolve 28 g anhydrous disodium phosphate and 40 g Rochelle salt in about 700 ml H_2O. Add 100 ml *N* sodium hydroxide and with

stirring add 80 ml 10% copper sulfate solution. Finally add 180 g anhydrous sodium sulfate. When dissolved, make to one liter and allow to stand for several days. The impurities settle out. Decant the clear top part of the solution, and filter the remainder through a good quality filter paper. The reagent keeps indefinitely.

Potassium iodate solution.—Dissolve 0.3567 g/1000 ml for 0.01 *N*.

Potassium iodide solution.—2.5%, stabilized with a drop or two of 25% NaOH.

Sulfuric acid.—2 *N*.

Sodium thiosulfate.—Make up 0.1 *N*, dilute to 0.005 *N* as used. Standardize against copper.

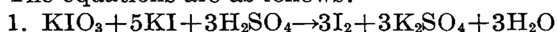
PROCEDURE

Pipette 5 ml of the Somogyi phosphate reagent into a 25x250 mm test-tube. Add 5 ml of sugar solution containing between 0.5 and 3.0 mg dextrose (in this particular series, a standard dextrose solution was made up to contain 100 mg/100 ml. Determinations were made on 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 mg dextrose.) For 0.5 mg/5 ml, measure by microburette 0.5 ml dextrose solution and 4.5 ml water. Obtain other concentrations in like manner with the appropriate amount of dextrose solution and make to 5 ml with water.

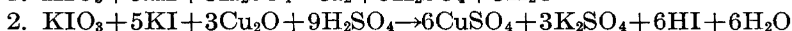
Introduce the test-tube into a rapidly boiling H_2O bath for 10 minutes, remove, and cool in ice water. (It has been found convenient to run five tubes at a time.) After cooling, add a quantity of KIO_3 which will yield sufficient I_2 to oxidize the Cu_2O. Add five ml of KIO_3 to the 0.5 and 1.0 mg samples; 10 ml to the 1.5 and 2.0 mg samples; and 15 ml to the 2.5 and 3.0 mg samples. (Somogyi offers the alternative of making up the copper reagent with the iodate in it. However, this necessitates having several lots of reagent in order to cover the range of concentrations of sugar.) Next add 1 ml of 2.5% KI for each 10 ml of KIO_3 present. Add the KI carefully down the sides of the test-tube to prevent mixing with the solution. Then acidify with 1.5 ml of 2 *N* H_2SO_4, adding it rapidly (a pipet with a broken tip was used) with shaking. After 2 minutes titrate with 0.005 *N* sodium thiosulfate solution, using starch indicator.

DISCUSSION

The equations are as follows:



1. $KIO_3+5KI+3H_2SO_4 \rightarrow 3I_2+3K_2SO_4+3H_2O$



2. $KIO_3+5KI+3Cu_2O+9H_2SO_4 \rightarrow 6CuSO_4+3K_2SO_4+6HI+6H_2O$

Equation 1 represents the blank titration, while equation 2 represents the reaction titration. Since HI is one of the products of the reaction when Cu_2O is dissolved in the acidic iodine solution, the amount of KI added is critical. An excess of KI shifts the equilibrium toward the left in equation 2, and causes a continuing reaction of the iodide with the iodate. In this case, the two equations are no longer analogous, and equation 1 does not represent the blank for equation 2.

Experimental results of 2 series of determinations run by one operator on different days is given in Table 1. With each group of 4 samples a water blank was run. This blank was titrated with either 5, 10, or 15 ml of added KIO_3, as applicable.

The copper-dextrose ratios obtained by this operator were considerably lower than those given by Somogyi. Some titration values are shown in Table 2.

TABLE 1.—*Results of two series of determinations by one operator*

ANALYST I	DATE	KIO_3 ADDED	TITER .005 *N* THIO	AVERAGE TITER	DEXTROSE PRESENT	DEXTROSE FOUND	DIFFERENCE
		ml	*ml*	*ml*	*mg*	*mg*	*mg*
Run 1	9/2/52	5	3.53		0.5	0.51	0.01
	9/2/52	5	3.49		0.5	0.50	0.00
Run 2	9/9/52	5	3.39		0.5	0.49	0.01
	9/9/52	5	3.45		0.5	0.50	0.00
				3.465			
Run 1	9/2/52	5	6.96		1.0	1.00	0.00
	9/2/52	5	7.08		1.0	1.01	0.01
Run 2	9/9/52	5	6.89		1.0	0.99	0.01
				6.98			
Run 1	9/2/52	10	10.45		1.5	1.50	0.00
	9/2/52	10	10.56		1.5	1.52	0.02
Run 2	9/9/52	10	10.33		1.5	1.48	0.02
	9/9/52	10	10.46		1.5	1.50	0.00
				10.45			
Run 1	9/2/52	10	14.02		2.0	2.01	0.01
	9/2/52	10	13.93		2.0	2.00	0.00
Run 2	9/9/52	10	13.92		2.0	2.00	0.00
	9/9/52	10	13.92		2.0	2.00	0.00
				13.95			
Run 1	9/2/52	15	17.15		2.5	2.51	0.01
Run 2	9/9/52	15	17.04		2.5	2.49	0.01
				17.095			
Run 1	9/2/52	15	20.44		3.0	3.00	0.00
	9/2/52	15	20.48		3.0	3.00	0.00
Run 2	9/9/52	15	20.50		3.0	3.00	0.00
				20.47			

Some of the factors probably contributing to the differences are variations in (1) test tube glass, (2) bath arrangement, (3) time required by individuals for titrating. Consequently, anyone using the method should always run sugar standards as well as water blanks in order to have a basis for calculation.

Another difficulty encountered was the discrepancy between duplicates run by the same operator. In some instances there were differences

TABLE 2.—*Titration values*

GLUCOSE	.005 *N* Cu AS REPORTED BY SOMOGYI	.005 *N* Cu THE AUTHOR
mg	*ml*	*ml*
0.5	3.70	3.46
1.0	7.40	6.98
2.0	14.80	13.95
3.0	22.20	20.47

as great as .11 and .13 ml of .005 *N* thiosulfate between duplicate samples run at the same time (Table 1). Average titrations on a given concentration of sugar varied by a similar amount from day to day. Table 3 shows the values obtained by one operator over a period of time on one concentration of dextrose. Two blanks were run simultaneously with each day's runs.

TABLE 3.—*Variation of titer with time*

DATE	DEXTROSE/5 ML	.005 *N* Cu
	mg	*ml*
Apr. 17, 1952	1.5	11.76
Apr. 17, 1952	1.5	11.70
Apr. 24, 1952	1.5	11.19
Apr. 24, 1952	1.5	11.14
Apr. 29, 1952	1.5	10.67
Apr. 29, 1952	1.5	10.70
Apr. 30, 1952	1.5	11.02
Apr. 30, 1952	1.5	11.04
May 2, 1952	1.5	11.25
May 2, 1952	1.5	11.19
May 9, 1952	1.5	10.71
May 9, 1952	1.5	10.70

Note: Iodate in Cu reagent. Data obtained from M. R. Dryden of the National Bureau of Standards.

From these preliminary studies it is apparent that further standardization of the method is in order. Therefore, it is recommended*—

(1) That further studies, including collaborative work, be initiated on Somogyi's phosphate method; and

(2) That comparative studies be made between Somogyi's phosphate and carbonate methods.

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REPORT ON TRANSMITTANCY OF SUGAR SOLUTIONS

BY F. W. ZERBAN (New York Sugar Trade Laboratory, New York, N. Y.)

In last year's work on this subject (1) it was found that two filtering materials, viz., specially prepared asbestos and Celite analytical filter aid, gave about the same average transmittancy results. On this basis the Celite was recommended as preferable because the filtration requires much less time and manipulation. Although each collaborator used the two filtering materials at about the same time, the entire work of each was carried out at different periods of the year, between January and June. As a consequence, the color of the sugars changed, as is well known from earlier work (2), and no conclusions could be drawn as to the reproducibility of results. It was therefore decided that this year all collaborators were to be requested to do their experimental work between February 10 and 20. It is very gratifying that all acceded to this request, and any effect of color change in the samples themselves was completely eliminated.

Three samples of raw sugar, a Cuban and a Puerto Rican supplied by S. M. Cantor, of the American Sugar Refining Company, and a Hawaiian furnished by T. R. Gillett, of the California and Hawaiian Sugar Refining Corporation, were distributed among seven collaborators, for whose co-

operation the writer is grateful. The following directions were sent to each of them:

Directions.—Three samples of raw sugar are being distributed, a Cuban, a Puerto Rican, and a Hawaiian. The transmittancy determinations of each of these samples are to be made as closely as possible between February 10 and 20, according to the following method:

The Celite to be used is the Celite Analytical Filter Aid of the Johns-Manville Corporation, marketed by Fisher Scientific Company, 711–723 Forbes Street, Pittsburgh, Pa. and 633–635 Greenwich Street, New York, N. Y. It is used as received. The solution of the raw sugar is prepared by placing 60 g of the sugar in a flask, adding 40 ml of distilled water, and rotating the flask until all the sugar is dissolved. Six g of the Celite is added to the sugar solution and the mixture is vigorously shaken for fifteen minutes. To insure complete separation of the first turbid runnings of the filtrate from the clear portion, without breaking the vacuum and thus disturbing the filter bed already formed, the fractional filtration apparatus of Sattler used in last year's work (1), is again employed. The Coors porcelain Büchner funnel (size 2, diameter of filtering plate 75 mm), is placed, by means of a 1-hole rubber stopper, on the top of a filtering tube which has a side arm near the top and a glass stopcock at the lower end. The side arm of the filtering tube is connected by means of the straight part of a T-tube with the side arm of the 250-ml filtering flask, and the side outlet of the T-tube is connected with the suction pump. After the filtering apparatus has been assembled, with the stopcock open, a circle of Schleicher & Schuell filter paper no. 589, blue ribbon, 7 cm. diameter, is placed on the filtering surface of the Büchner funnel, wetted with water, and the excess water sucked down by vacuum through the filtering tube into the filtering flask underneath. The stopcock is now closed, and the well-shaken mixture of sugar solution and Celite is poured evenly over the filter paper. About 5 to 10 ml of the filtrate, which is somewhat turbid, is collected in the filtering tube, and then run into the filtering flask by opening the stopcock. The stopcock is closed again, and two or three more portions of 5 to 10 ml filtrate are collected as previously in the filtering tube, then run down into the filtering flask to wash the inner wall of the filtering tube free of any small particles of turbidity. It is essential that during the entire filtration process the bed of Celite be kept covered with sugar solution and not allowed to run dry. The final clear filtrate is collected in the filtering tube and transferred to a small bottle in which it is thoroughly mixed. The refractometer Brix is determined, and the concentration c (grams dry substance per ml solution) is calculated by multiplying the Brix by the corresponding true density and dividing by 100. The remainder of the solution is used for the transmittancy determinations.

It is presupposed that the collaborators are versed in spectrophotometry and familiar with the exacting optical cleanliness required. The wave length and the transmission scales should be checked, preferably with a standard glass filter of the National Bureau of Standards. The cell thickness should preferably be varied so that the readings fall as much as possible in the range of 25 to 75 per cent transmittancy to avoid large percentage errors in the corresponding absorbancy indexes.

The transmittancies are to be determined at six wave lengths, 375, 420, 480, 560, 640, and 720 mμ, with distilled water as the 100% transmittancy standard.

Three separate complete tests of each of the three sugars are to be made, each of the nine tests starting with the solid raw sugar. The details of the procedure described should be strictly adhered to in order to attain the closest possible agreement between the results for each of the three sugars.

The following data are to be reported for each of the nine series of determinations:

(1) The observed *transmittancy* at each of the six wave lengths, *and* the *cell thickness* used for each of the transmittancy measurements.

(2) The *concentration* c of each of the nine final filtrates used for the measurements, in grams of solids per milliliter of solution, calculated as described above.

(3) The type of spectrophotometer used.

Reports have been received from all the seven collaborators who used the instruments and experimental conditions specified below, all of them between February 10 and 20:

(1) T. R. Gillett, California and Hawaiian Sugar Refining Corp., Crockett, Calif. Beckman DU instrument, 1 cm. cell, $c=0.744$ to 0.767; also photoelectric filter photometer of own design, cells of 0.5 and 1 cm., $c=0.756$ to 0.765.

(2) C. A. Fort, Southern Regional Research Laboratory, New Orleans, La. Beckman DU instrument, cells of 0.5, 1.0, and 2.0 cm., $c=0.780$ to 0.787.

(3) Carl Erb, New York Sugar Trade Laboratory, New York, N. Y. Beckman B instrument, cells of 0.1, 0.25 and 0.52 cm., $c=0.768$ to 0.786.

(4) James Martin, New York Sugar Trade Laboratory, New York, N. Y. Same instrument and cells used as by Carl Erb, $c=0.770$ to 0.779.

(5) R. Winston Liggett, American Sugar Refining Company Research Laboratory, Philadelphia, Pa. Beckman DU instrument, cells of 0.096 and 0.999 cm., $c=0.777$ to 0.865.

(6) V. R. Deitz, National Bureau of Standards, Washington, D. C. Beckman DU instrument, cells of 0.05, 0.2, and 1 cm., $c=0.767$ to 0.793.

(7) R. T. Balch, U. S. Department of Agriculture, Houma, La. Coleman Jr. Instrument; cells of 0.1 and 0.8 cm., $c=0.756$ to 0.776.

RESULTS

Table 1 gives the absorbancy indexes, a, reported by all the collaborators.

The table shows that the results of collaborators 1 to 4, using the Beckman DU and Model B instruments, are in fair agreement. Those of 3 and 4 are slightly higher than those of 1 and 2, but it was found later that this was due to the structural details of the instruments used. The Beckman B is equipped for the use of 20 cm cells without change in the location of the holder for cells of 1 cm or less thickness. When the instrument was used with such cells for raw sugar solutions, they were at a distance of about 12 cm from the photocell, instead of close to it. As is well known this condition causes even the small amounts of scattered light of filtered raw sugar solutions to produce a slightly lower transmittancy than normally. In a few experiments the transmittancy near the center of the scale was increased 0.4 to 0.8 per cent by placing the absorption cell near the photocell; this corresponds to a decrease in the absorbancy index of the Hawaiian sugar from 1.311 to 1.295 at 480 mμ, and from 0.589 to 0.565 at 560 mμ. With the absorption cell at a normal distance from the photocell, the results of collaborators 3 and 4 would have approached the results of collaborators 1 and 2 more closely, and the agreement between the four results would have been even better.

TABLE 1.—*Absorbancy indexes*

COLLABR.	1	2	3	4	5	6	7
INSTRUMENT	BECKMAN DU	CARY	BECKMAN B	BECKMAN B	BECKMAN DU	BECKMAN DU	COLEMAN JR.
Wave-length				*Cuban Sugar*			
375 mμ							
No. 1	—	—[a]	10.797	12.052	11.391	8.615	—
No. 2	—	5.353	11.527	12.243	10.773	8.553	—
No. 3	—	5.353	—	12.920	11.527	8.481	—
Av.		5.353	11.162	12.405	11.231	8.550	
420 mμ							
No. 1	4.835	4.499[b]	4.680	4.606	4.453	3.788	4.399
No. 2	4.854	4.047	4.576	4.617	4.323	3.689	4.138
No. 3	4.906	4.182	4.541	4.746	4.537	3.719	4.183
Av.	4.865	4.243	4.599	4.656	4.438	3.732	4.240
480 mμ							
No. 1	2.159	2.223	2.233	2.197	2.057	1.823	2.113
No. 2	2.178	2.111	2.200	2.220	1.981	1.764	2.078
No. 3	2.186	2.118	2.215	2.332	2.144	1.784	2.091
Av.	2.174	2.144	2.216	2.250	2.061	1.790	2.094
560 mμ							
No. 1	0.980	1.001	1.025	0.991	0.884	0.832	0.937
No. 2	1.000	0.975	0.992	1.018	0.864	0.812	0.939
No. 3	0.995	0.983	0.997	1.087	0.973	0.816	0.938
Av.	0.992	0.986	1.005	1.032	0.907	0.820	0.938
640 mμ							
No. 1	0.493	0.502	0.529	0.495	0.473	0.416	0.498
No. 2	0.503	0.485	0.509	0.511	0.458	0.405	0.501
No. 3	0.489	0.492	0.493	0.538	0.490	0.408	0.501
Av.	0.495	0.493	0.510	0.515	0.474	0.410	0.500
720 mμ							
No. 1	0.217	0.220	0.242	0.215	0.206	0.171	—
No. 2	0.219	0.211	0.209	0.216	0.200	0.165	—
No. 3	0.215	0.213	0.207	0.241	0.206	0.170	—
Av.	0.217	0.215	0.224	0.219	0.204	0.169	—

[a] Transmittancy read below 1%.
[b] Transmittancy read below 3%.

TABLE 1—(*continued*)

COLLABR.	1	2	3	4	5	6	7
INSTRUMENT	BECKMAN DU	CARY	BECKMAN B	BECKMAN B	BECKMAN DU	BECKMAN DU	COLEMAN JR.
Wave-length				*Puerto Rican Sugar*			
375 mμ							
No. 1	—	—[a]	14.892	14.712	14.731	10.858	—
No. 2	—	5.399	14.859	14.772	14.757	11.187	—
No. 3	—	5.708	—	14.778	14.974	10.916	—
Av		5.554	14.876	14.754	14.821	10.987	
420 mμ							
No. 1	—	—[b]	7.500	6.711	6.616	4.834	5.476
No. 2	—	4.935	6.787	6.821	6.543	4.969	5.904
No. 3	—	5.223	6.725	6.747	6.627	4.8 99	5.941
Av.		5.079	7.004	6.760	6.595	4.901	5.774
480 mμ							
No. 1	3.324	3.264	3.379	3.382	3.202	2.729	2.894
No. 2	3.233	3.158	3.425	3.402	3.179	2.744	3.164
No. 3	3.266	3.196	3.393	3.442	3.240	2.744	3.179
Av.	3.274	3.206	3.399	3.409	3.207	2.739	3.079
560 mμ							
No. 1	1.369	1.354	1.388	1.378	1.278	1.125	1.173
No. 2	1.335	1.321	1.387	1.401	1.264	1.125	1.262
No. 3	1.338	1.331	1.416	1.417	1.316	1.137	1.270
Av.	1.347	1.335	1.397	1.399	1.286	1.129	1.235
640 mμ							
No. 1	0.585	0.585	0.613	0.630	0.596	0.465	0.587
No. 2	0.573	0.579	0.625	0.625	0.587	0.464	0.590
No. 3	0.568	0.579	0.607	0.631	0.604	0.484	0.595
Av.	0.575	0.581	0.615	0.629	0.596	0.471	0.591
720 mμ							
No. 1	0.245	0.242	0.256	0.232	0.232	0.189	—
No. 2	0.246	0.240	0.230	0.275	0.271	0.187	—
No. 3	0.241	0.237	0.228	0.278	0.254	0.193	—
Av.	0.244	0.240	0.238	0.262	0.252	0.190	—

[a] Transmittancy read below 1%.
[b] Transmittancy read below 2%.

TABLE 1—(*continued*)

COLLABR.	1	2	3	4	5	6	7
INSTRUMENT	BECKMAN DU	CARY	BECKMAN B	BECKMAN B	BECKMAN DU	BECKMAN DU	COLEMAN JR.
Wave-length				*Hawaiian Sugar*			
375 mμ							
No. 1	4.972	2.559[a]	7.029	6.723	6.288	4.671	—
No. 2	4.835	2.675	6.766	6.662	6.448	4.654	—
No. 3	4.823	2.675	6.702	6.895	6.420	4.847	—
Av.	4.877	2.636	6.832	6.760	6.385	4.724	
420 mμ							
No. 1	2.750	2.676	2.865	2.913	2.564	2.261	2.478
No. 2	2.817	2.633	2.879	2.833	2.684	2.205	2.539
No. 3	2.810	2.621	2.861	2.963	2.663	2.271	2.539
Av.	2.792	2.643	2.868	2.903	2.637	2.246	2.519
480 mμ							
No. 1	1.278	1.270	1.328	1.327	1.153	1.022	1.175
No. 2	1.295	1.258	1.326	1.283	1.206	0.986	1.192
No. 3	1.292	1.254	1.318	1.365	1.180	1.018	1.192
Av.	1.288	1.261	1.324	1.325	1.180	1.009	1.186
560 mμ							
No. 1	0.577	0.569	0.594	0.595	0.532	0.456	0.531
No. 2	0.575	0.555	0.583	0.567	0.541	0.443	0.529
No. 3	0.574	0.545	0.579	0.593	0.541	0.455	0.525
Av.	0.575	0.556	0.585	0.585	0.538	0.451	0.528
640 mμ							
No. 1	0.277	0.280	0.284	0.244	0.250	0.213	0.265
No. 2	0.271	0.270	0.258	0.266	0.251	0.208	0.268
No. 3	0.269	0.263	0.275	0.290	0.257	0.214	0.264
Av.	0.272	0.271	0.272	0.267	0.253	0.212	0.266
720 mμ							
No. 1	0.116	0.117	0.116	0.115	0.101	0.083	—
No. 2	0.111	0.109	0.094	0.093	0.104	0.080	—
No. 3	0.112	0.108	0.112	0.116	0.093	0.082	—
Av.	0.113	0.111	0.107	0.102	0.099	0.082	—

[a] Transmittancy read below 1%.

Collaborator 5 (Beckman DU) obtained generally lower results than collaborators 1 to 4, and collaborator 6, with the same type of instrument, the lowest of all. Collaborator 7, with a Coleman Junior instrument, also obtained generally low results. Inquiry among the collaborators revealed that all of them followed the directions in every detail except that collaborator 5 dissolved the raw sugars in hot water instead of in water at room temperature. It is therefore possible that the differences in the results were due to unforeseen causes, such as differences in the quality of the Celite analytical filter aid used, differences in the vacuum applied, and other minor details.

Reproducibility of results is shown in Table 2, which gives the difference between the maximum and the minimum of the three results, in per cent of the average of the three results, by each observer. The values

TABLE 2.—*Difference between maximum and minimum results of each individual triplicate set, in per cent of average result of the triplicate set*

WAVELENGTH	COLLABORATOR							AVERAGE
	1	2	3	4	5	6	7	
mμ				*Cuban Sugar*				
420	1.5	10.7	3.0	3.0	4.8	2.7	6.2	4.6
480	1.2	4.3	1.5	6.0	7.9	3.3	1.7	3.7
560	2.0	2.6	3.3	9.3	12.0	2.4	0.2	4.5
640	2.8	3.4	7.1	8.3	6.8	2.7	0.6	4.5
720	1.8	4.2	16.0	11.6	2.9	3.6	—	6.7
				Puerto Rican Sugar				
420	—	5.7	11.1	1.6	1.3	2.7	8.1	5.1
480	2.8	3.3	1.4	1.8	1.9	0.5	9.2	3.0
560	2.5	2.5	2.1	2.8	4.0	1.1	7.7	3.4
640	3.0	1.0	2.9	1.0	2.9	4.2	1.4	2.3
720	2.0	2.1	11.8	17.2	15.0	3.2	—	8.6
				Hawaiian Sugar				
420	2.4	2.1	0.6	4.5	4.6	2.9	2.4	2.8
480	1.3	1.3	0.8	6.2	4.5	3.6	1.4	2.7
560	0.5	4.3	2.6	4.8	1.7	2.9	1.1	2.6
640	2.9	6.3	9.6	17.2	2.4	2.8	1.5	6.1
720	4.4	8.1	20.6	21.3	11.1	3.7	—	11.5
				Mean for the three sugars				
420	2.0	6.2	4.9	3.0	3.6	2.8	5.6	4.2
480	1.8	3.0	1.2	4.7	4.8	2.5	4.1	3.1
560	1.7	3.1	2.7	5.6	5.9	2.1	3.0	3.5
640	2.9	3.6	6.5	8.8	4.0	3.2	1.2	4.3
720	2.7	4.8	16.3	16.7	9.7	3.5	—	8.9

obtained at 375 mμ are omitted from consideration because of their irregularity.

The table shows that the discrepancies in the results of each individual observer are fairly high. At 480 and 560 mμ they range from 2.6 to 4.5 per cent and average below 4 per cent, but at 720 mμ they average nearly 9 per cent, ranging from 6.7 to 11.1 per cent. It is doubtful whether these deviations can be greatly improved.

TABLE 3.—*Standard error of individual results*

WAVELENGTH	COLLABORATORS 1 THROUGH 7			COLLABORATORS 1 THROUGH 4		
	MEAN a	STANDARD ERROR IN a	STANDARD ERROR IN PER CENT	MEAN a	STANDARD ERROR IN a	STANDARD ERROR IN PER CENT
			Cuban Sugar			
420	4.396	0.365	8.3	4.591	0.260	5.7
480	2.104	0.151	7.2	2.196	0.057	2.6
560	0.955	0.073	7.7	1.004	0.030	3.0
640	0.463	0.042	9.2	0.503	0.016	3.3
720	0.208	0.021	10.1	0.219	0.011	5.2
			Puerto Rican Sugar			
420	6.074	0.858	14.1	6.431	0.877	13.6
480	3.188	0.225	7.1	3.322	0.096	2.9
560	1.304	0.075	5.8	1.370	0.034	2.4
640	0.580	0.049	8.5	0.600	0.024	4.0
720	0.238	0.027	11.3	0.246	0.016	6.6
			Hawaiian Sugar			
420	2.658	0.219	8.3	2.802	0.110	3.9
480	1.225	0.108	8.8	1.300	0.034	2.6
560	0.546	0.024	4.4	0.576	0.015	2.7
640	0.259	0.016	6.4	0.271	0.012	4.5
720	0.103	0.010	9.7	0.110	0.008	7.5

Note. Figures for all collaborators are based on 21 individual results each at all wavelengths except 720 mμ (18 results for each sugar) and 420 mμ (17 results for Puerto Rican sugar) .The figures for 4 collaborators are based on 12 individual results each, at all wavelengths except 420 mμ for the Puerto Rican sugar (8 results). The mean standard error, in per cent, for all three sugars is:

Wave length, mμ	420	480	560	640	720
7 Collaborators	10.2	7.7	6.0	8.0	10.4
4 Collaborators	7.7	2.7	2.7	3.9	6.4

In order to obtain reliable information on over-all reproducibility of results the standard error of estimate of the results obtained in all the single (not average) observations of all collaborators has been calculated by the formula $\sqrt{(S)(n-1)}$, where S is the sum of the squares of the individual deviations from the mean, and n is the number of individual results. The results are given in Table 3 for all the collaborators, and also for collaborators 1 through 4 who obtained results in fair agreement.

The mean standard error of the four collaborators who showed fair agreement in the results is only about half of that for all collaborators.

At wave length 560 mμ, for which the absorbancy index may be considered equivalent to the color of the solution of the sugar the standard error is less than 3 per cent for collaborators 1 through 4 or about as low as the error of individual tests by one operator. But a standard error of 6 per cent at 560 mμ, as shown by all seven collaborators, is too high to be acceptable for an official method, and further collaborative work will have to be carried out to obtain results in better agreement. The effect of the quality of individual lots of Celite analytical filter aid will have to be eliminated by making tests with portions of a single lot furnished to all collaborators. The degree of vacuum used in the filtration of the sugar solutions will have to be standardized, and the *p*H of the filtered solution will have to be considered. It is hoped that with improvements in experimental details better agreement between collaborators will be achieved. A. B. Cummins, of the Johns-Manville Corporation, who on request of the writer has made some tests by the filtration method specified in this year's directions, has suggested that the sugar be dissolved in water that has been heated to the boiling point, but without further application of heat, that the quantity of Celite be reduced from 6 to 3 grams per sample, that the time of shaking the Celite with the sugar solution be reduced from fifteen minutes to about one minute, and that two portions of 10 ml each of filtrate be discarded before the portion for transmittancy determination is collected.

TABLE 4.—*Results with a photoelectric filter photometer**

COLOR FILTER NO.	1	2	3	4
Approximate dominant wave length (mμ)	420	535	585	640
Cuban Sugar, 0.762 g/ml	2.517	1.231	0.706	0.400
P. R. Sugar, 0.756 g/ml	3.397	1.698	0.865	0.458
Hawaiian Sugar, 0.765 g/ml	1.725	0.758	0.421	0.224

* Filters:
1. Blue (Corning #554, 4 mm.)
2. Green (Corning #401, 4 mm.)
3. Yellow (Corning #351 & 398, 2 mm. each)
4. Red (Corning #243, 4 mm.)

The results with the photoelectric filter photometer (Collaborator 1) are shown in Table 4. Comparison with the corresponding figures in Table 1 shows that, like last year, the effective wave length of the blue filter is very much higher than 420 but below 480 mμ, that of the green and yellow filters is somewhere near correct, and that of the red filter is a little above 640 mμ.

RECOMMENDATIONS

It is recommended* that this year's work on the reproducibility of the

* For report of Subcommittee D and action of the Association, see *This Journal*, 36, 64 (1953).

Celite filtration method be repeated with a more exact standardization of the procedure.

REFERENCES

(1) ZERBAN, F. W., *This Journal*, **35**, 636 (1952).
(2) FOWLER, A. P., and KOPFLER, F. W., *Proc. Cuban Sugar Technol. Assoc.*, **9**, 210 (1935).

REPORT ON REDUCING SUGAR METHODS

By EMMA J. MCDONALD (National Bureau of Standards, Washington 25, D. C.), *Associate Referee*

Last year the deWhalley method for the determination of invert sugar in refined white sugars was studied. The method was found to be rapid and to give reproducible results. However, it was found that some modification of the color standards was necessary in order to compensate for unavoidable differences that occur in different laboratories. Unless there is evidence of interest in this method, the Associate Referee will not consider it further for the purposes of this Association.

The method of Lane and Eynon is probably most generally used in the macro analysis of reducing sugars. The sugar solution is added by means of a burette to boiling Fehling solution. The end point is determined by the addition of methylene blue. Two similar modifications of this method have been developed for use when solutions containing small amounts of invert sugar are to be analysed. A fixed amount of the unknown solution is added to the Fehling solution and the titration is completed by standard invert sugar. A control titration is run in which the standard invert sugar is added to a known volume of a sucrose solution. The concentration of sucrose in this solution is similar to that in the unknown. These modifications have been used for beet diffusion juices and for cane sirups, molasses, and low grade sugars.

Another modification of the Lane and Eynon method is known as the constant volume modification. This is applicable when the reducing sugar content is approximately known and can therefore be properly adjusted. The essential feature is that the volumes of sugar solution required for titration are within a narrow range and a single factor is therefore applicable to all titrations. This modification is used in this country and Great Britain in cases where sucrose is not present. It would be well to have collaborative work done on these two modifications during the following year.

The concentrations of the standard invert sugar solution and of the sucrose solution used in the blank titration when determining a small quantity of invert in the presence of sucrose by the first modification, and of the standard reducing sugar solution used in the constant volume modification, depend upon the product being analysed. For collaborative

work a detailed procedure applicable to the samples under consideration must be supplied.

It is recommended* that collaborative work be done on the volumetric determination of small amounts of invert sugar by the Lane and Eynon method and reducing sugars by the constant volume modification of the same method.

It is recommended that the study on reducing sugar methods, including chromatographic procedures, be continued.

REPORT ON DENSIMETRIC AND REFRACTOMETRIC METHODS FOR SUGAR PRODUCTS

By CARL F. SNYDER (National Bureau of Standards, Washington 25, D. C.), *Associate Referee*

At the 1951 meeting of the Association, Dr. F. W. Zerban presented a paper by Zerban and Martin on the refractive indices of raffinose hydrate solutions. From the data obtained in this work, the authors derived an equation relating per cent raffinose hydrate by weight and refractive index. A recommendation was made that the Association adopt this table. In the course of the discussion, the question arose regarding the desirability of carrying out collaborative work to confirm these values. This procedure did not appear necessary in view of the painstaking care exercised by the authors, as shown by an analysis of their data, but it was suggested that a number of points be checked by another laboratory. This was done through the cooperation of two of the Associate Referees, E. J. McDonald and B. K. Goss.

In the course of their investigation of the optical rotation of raffinose hydrate at different concentrations, the refractive index of each of the solutions was measured. The instrument employed was a carefully calibrated Bausch and Lomb precision refractometer and independent measurements were made by two observers. Eight concentrations were measured at 20°C., and the values obtained were in agreement with the values of Zerban and Martin. In addition, five of the solutions were measured at 25°C. The temperature coefficient calculated from the differences in refractive indices at 20° and 25°C. was found to be 10×10^{-5} for the range 0 to 5 per cent raffinose hydrate and 11×10^{-5} for the range 5 to 13 per cent. This increase is in agreement with the refractive index temperature coefficients for sucrose and levulose. Therefore it is recommended† that the Zerban and Martin table of refractive indices of raffinose hydrate be made official.

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* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 64 (1953).
† For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 64 (1953).

McDonald and Goss also determined the densities of raffinose hydrate solutions and calculated the equations for both true and apparent densities. By means of these equations a table of density values for the range 1 to 15 per cent raffinose hydrate has been computed.

The above investigation on raffinose hydrate is a part of a collaborative study by the U. S. National Committee of the International Commission for Uniform Methods for Sugar Analysis (1). The detailed results will be published separately.

Dr. Zerban announced last year that the New York Sugar Trade Laboratory proposed to determine the refractive indices of sucrose solutions in the range 60 to 70 per cent. The present accepted values are open to question for concentrations from 66 to 71 per cent, since they were obtained by a graphic extrapolation (1). Mr. James Martin of the above laboratory measured sixteen concentrations and obtained values deviating somewhat from the published table. Similar measurements were subsequently made at the National Bureau of Standards by the Referee and his associate, M. R. Dryden, at twelve different concentrations. In both laboratories the deviations were somewhat larger than anticipated and reflect the difficulties encountered in refractive index measurements on sugar solutions of high concentrations. The work is being continued with the view of obtaining sufficient data to permit a revision of the present official table.

REFERENCE

(1) Proceedings I.C.U.M.S.A., 10th session, *Intern. Sugar J.*, **52**, No. 618, 619 (1950).

No report was given on: drying methods; honey; corn sirup and corn sugar; or on starch conversion products.

The contributed paper entitled "The Sugar Content of Hydrol (Corn Feeding Molasses)," by G. T. Peckham, Jr., and C. E. Engel, appears on p. 455.

REPORT ON CACAO PRODUCTS

By W. O. WINKLER (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Referee*

Work done on cacao products was very limited this year. No report was received on the determination of maltose in cacao products, but the Associate Referee is experimenting with the chromatographic sugar method of Dr. E. MacDonald and hopes to have the problem resolved by next year. No report was received on the determination of lactose in cacao products containing other reducing sugars and no work was done this

year on characteristic cacao ingredients (theobromine, cacao red, etc.).

A revision in the method for lecithin made further collaborative work necessary. The results obtained by the collaborators on the revised procedure agree reasonably well, and the Referee is in accord with the recommendations of the Associate Referee that the method be adopted, first action.

PECTIC ACID

The only collaborative work done this year on the method for pectic acid was conducted by a group of British chemists. The Referee received information on September 22, 1952, that a report will be forthcoming; at this writing it has not been received. Some study was made last year by a British group, but their report was not received until after last year's A.O.A.C. meeting.

In contrast to our experience, they have had trouble with the first filtration of the pectic acid solution in dilute NH_4OH, following the first hydrolysis and precipitation with the aid of Celites. The average pectic acid value reported by all the chemists is in the general range, although a little higher than found here on similar samples containing the same quantity of shell. Agreement among collaborators was not so good as obtained in the work done here. This may be partially due to lack of experience with the method.

It is hoped that the reasons for the slow rate of filtration they experienced and any other troubles may be ascertained and eliminated through cooperative efforts.

RECOMMENDATIONS

It is recommended*—

(1) That the work on methods for the determination of maltose in cacao products be continued.

(2) That work on the determination of lactose in cacao products containing other reducing sugars be continued.

(3) That the revised method for lecithin in cacao products, studied collaboratively this year, be adopted, first action.

(4) That the study of characteristic cacao constituents such as cacao red, theobromine, etc., be continued.

(5) That work on the hydrolytic colorimetric method for pectic acid in cacao products and work on the determination of pectic acid in milk-containing products be continued.

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 61 (1953).

REPORT ON LECITHIN IN CACAO PRODUCTS

By J. H. BORNMANN (Food and Drug Administration, Federal Security Agency, Chicago 7, Illinois), *Associate Referee*

The Associate Referee's report of last year* included a revision of the proposed method which involved a simplification of the extraction procedure and a choice of official micro methods for P_2O_5 determination. Committee D felt that the changes were more than editorial, and recommended that the revised method be studied collaboratively.

Two samples of commercial sweetened milk chocolate, prepared for analysis by the writer, were sent to collaborators with the request that the revised extraction procedure be used and that P_2O_5 be determined by both **20.49–20.50** (Method I) and **6.39–6.40** (Method II). Sample 1 declared added lecithin whereas Sample 2 did not. Collaborative results are given in Table 1.

TABLE 1.—*Lecithin in sweetened milk chocolate*

ANALYST	SAMPLE 1		SAMPLE 2	
	METHOD I	METHOD II	METHOD I	METHOD II
	per cent	*per cent*	*per cent*	*per cent*
1	0.289	0.314	0.098	0.115
	0.299	0.287	0.097	0.011
2	0.300[1]	0.300	0.107[1]	0.114
	0.300[1]	0.300	0.107[1]	0.114
	0.309[2]		0.114[2]	
	0.307[2]		0.109[2]	
3	0.47	0.49	0.25	0.29
	0.48	0.50	0.28	0.27
4	0.299	0.355	0.111	0.136
	0.299	0.355	0.110	0.129
5	0.312	0.330	0.158	0.130
	0.337	0.307	0.146	0.136
6	0.30	—	0.12	—
	0.27		0.11	
7	0.275	0.305	0.095	0.098
	0.300	0.305	0.091	0.092

[1] Using a spectrophotometer.
[2] Using a neutral wedge photometer.

* *This Journal*, **35**, 656 (1952).

None of the collaborators reported any difficulty with Method I. Perlmutter suggested ashing in the beaker (Method II) rather than transferring to a crucible. The Associate Referee sees no objection to this proposal, and it might be well to add a sentence to the proposed Method II: "Ashing may be done in the beaker in which the extract is evaporated."

The Associate Referee is indebted to the following collaborators:

Sam H. Perlmutter, Minneapolis District, Food and Drug Administration.
Aldrich F. Ratay, Cincinnati District, Food and Drug Administration.
Frank P. Colten, Walter Baker Chocolate & Cocoa Division, General Foods Corporation, Dorchester, Mass.
E. Borker, General Foods Corporation, Hoboken, New Jersey.
Mathew L. Dow, St. Louis District, Food and Drug Administration.
F. E. Yarnall, Kansas City District, Food and Drug Administration.
John H. Bornmann, Chicago District, Food and Drug Administration.

RECOMMENDATIONS*

It is recommended—

(1) That the following sentence be added to proposed Method II: "Ashing may be done in the beaker in which the extract is evaporated."

(2) That the proposed method, as amended, be allowed to remain first action, and that no further work be done until a method is found which is capable of distinguishing cacao lecithin from lecithin obtained from another source.

No report was made on malt solids, pectic acid, cacao ingredients, or lactose.

REPORT ON FRUITS AND FRUIT PRODUCTS

By R. A. Osborn (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Referee*

The Associate Referee has revised and clarified the procedure for tartaric acid. Precipitation, filtration, and washing of the potassium bitartrate precipitate occurs at or near 0°C. The collaborative results obtained by four collaborators with 2 samples containing less than 50 mg of tartaric acid in the aliquots taken for analysis indicate satisfactory recoveries with the revised procedure and good agreement between all collaborators.

Three analysts submitted collaborative results for *l*-malic acid on three samples. Using the revised procedure for tartaric acid, the samples

* For report of Subcommittee D and action of the Association, see *This Journal*, 36, 61 (1953).

were first treated to remove pectin and tartaric acid, and the filtrates were concentrated for the determination of *l*-malic acid. The results obtained by the collaborators are satisfactory. The Associate Referee stresses the importance of protecting the solution from light during and after treatment with uranium acetate.

In *Methods of Analysis*, 7th Ed., **20.39, 20.40,** and **20.41** the procedure for *l*-malic acid provides for the interference of isocitric acid. The procedure studied this year is suitable only in the absence of isocitric acid. Three of the organic acids which may be present in fruits are known to be optically active, viz., tartaric, *l*-malic, and isocitric. Before *l*-malic acid can be determined by a polarization procedure it is necessary first to remove the interfering tartaric and isocitric acids when they are present.

Further study of the procedure for the removal of isocitric acid prior to the determination of *l*-malic acid by polarization has been recommended. It is pointed out that only the blackberry is known to contain isocitric acid; nevertheless the analyst who has an unknown sample of fruit or fruit product cannot assume that it is free from isocitric acid. On the other hand, there may be instances where the analyst can be certain that blackberry is absent and prior precipitation and removal of isocitric acid would be unnecessary.

The Referee concurs with the recommendation of the Associate Referee.

It is recommended*—

1. That the two lines in the procedure for the determination of tartaric acid, *This Journal*, **34,** 75, beginning "dilute 20 ml . . . " be deleted.

2. That the first action method for tartaric acid, **20.35–20.36,** as redrafted in this year's report, be adopted as official.

3. That the procedure for *l*-malic acid, applicable in the absence of iso citric acid, as given in this year's report be adopted as an alternate procedure, first action.

4. That study of methods for the determination of fruit acids be continued.

5. That study of methods for the examination of frozen fruits for fruit, sugar, and water content be continued.

6. That study of methods for the determination of fill of container for frozen fruits be continued.

* For report of Subcommittee D and action of the Association, see *This Journal*, **36,** 63 (1953).

REPORT ON FRUIT (TARTARIC AND LAEVO-MALIC) ACIDS

By L. W. FERRIS (Food and Drug Administration, Federal Security Agency, Buffalo 3, N. Y.), *Associate Referee*

TARTARIC ACID

In the 1951 report on tartaric acid (1) some results were low and it was recommended that study of the method be continued. Some changes in details of manipulation were made to insure more complete precipitation of the potassium bitartrate and to prevent its redissolving during filtration and washing. The method as redrafted is as follows:

TOTAL TARTARIC ACID—BITARTRATE METHOD

REAGENTS

(a) *Lead acetate soln.*—Dissolve 75 g normal Pb acetate in H_2O, add 1 ml acetic acid, and dil. to 250 ml.

(b) *Potassium hydroxide.*—30%.

APPARATUS

Device for filtering at 0°.—Use app. similar to that described under **16.17(d)**.

PREPARATION OF SAMPLE AND REMOVAL OF PECTIN

Take a quantity of sample prepd as described under **20.2** having titratable acidity approximating 3 ml *n* acid with solids content not over 20 g. Designate as "*A*" the ml of *N* alkali required to neutralize sample. Adjust vol. of sample to ca 35 ml by evapn or by addn of H_2O, add 3 ml of *N* H_2SO_4 and heat to 50°. Pour adjusted sample into 250 ml volumetric flask, rinse with 10 ml hot H_2O, and finally with alcohol, cool, make to mark with alcohol, shake, and let stand until pptd pectin separates, leaving a clear liquid, overnight if necessary. Transfer to centrifuge bottle, add 0.2 g filter aid, shake vigorously, centrifuge, and decant thru a retentive paper. Cover funnel with watch glass to prevent evapn. Pipette 200 ml of filtrate into centrifuge bottle.

If original sample is alc., it may contain esters of organic acids, and saponification is necessary. Adjust vol. to 35 ml, add "*A*" + 3 ml *N* KOH, heat to ca 60°, and allow to stand overnight. Add "*A*" + 6 ml of *N* H_2SO_4, transfer to 250 ml volumetric flask, and proceed as directed above.

DETERMINATION

To soln in centrifuge bottle add vol. of Pb acetate soln **(a)** equal to "*A*" + 3 ml, or in case saponification was made, "*A*" + 6 ml, 0.2 g filter aid, shake vigorously 2 min. and centrifuge. Test supernatant liquid with a few drops of Pb acetate soln and if a ppt is formed, add more of the Pb acetate soln, shake, and again centrifuge. Decant and allow to drain thoroly by inverting the bottle several min. To material in centrifuge bottle add 50 ml 80% alcohol, shake vigorously to disperse the ppt, add 150 ml more 80% alcohol, shake, centrifuge, decant, and drain. To Pb salts in centrifuge bottle add ca 150 ml H_2O, shake thoroly, and pass in H_2S to satn. Unsatn is indicated by the presence of a partial vacuum obtained by stoppering bottle, shaking, and observing partial vacuum when carefully removing stopper. Transfer to 250 ml volumetric flask, dil. to mark with H_2O, and filter thru folded paper.

Transfer 100 ml of clear filtrate to a 250 ml I flask, tared with 2 or 3 glass beads. (A Harvard trip balance sensitive to 0.1 g is convenient.) Evap. on gauze over flame to ca 30 ml, remove from flame, add a second 100 ml aliquot, and evap. to 19 g (±0.5 g). Neutralize with 30% KOH, one drop at a time, using phenolphthalein indicator, and add *one* drop in excess. Add 2 ml acetic acid, 0.2 g of filter aid (Celite 545 is satisfactory), and (slowly with agitation 80) ml of 95% alcohol. Cool in cracked ice-salt mixt., shake vigorously 2 min., place in refrigerator and hold overnight at 0°C. Cover the filtering disc (see "Apparatus") with a thin layer of asbestos and place over it a thin layer of filter aid. Place cracked ice in the outer funnel, wash the filter mat with ice-cold alcohol and allow to stand a few min. to cool the filter thoroly. Swirl the flask to suspend the filter aid and ppt, and filter at 0°, sucking the mat dry. (Filtrates and washings should be used for *l*-malic acid detn.) Wash the stopper with ca 15 ml ice-cold 80% alcohol, allowing the wash liquid to run into the pptn flask. Stopper and shake to wash the flask well. A stirring rod bent at a 45° angle 1″ from end helps in washing the inside of the filter tube. Conduct the wash liquid completely around the inside of the filter tube and suck dry. Wash flask and filter tube with two 15 ml portions each of ice-cold 80% alcohol. While filtering, keep the flask cold with cracked ice. Remove ice from outer funnel, and transfer ppt and pad to pptn flask with boiling CO_2 free H_2O. Heat almost to boiling, and titrate with 0.1 *N* alkali, using phenolphthalein indicator. 1 ml 0.1 *N* alkali = 0.015 g tartaric acid. Tartaric acid ÷ 0.64 = tartaric acid in sample taken.

Using the above procedure, two samples of apple juice containing small amounts of added tartaric acid were examined by 4 analysts. The apple juice, with nothing added, showed 0.012 g/100 ml of tartaric acid. Results are shown in Table 1.

TABLE 1.—*Grams tartaric acid/100 ml*

ANALYST	SAMPLE NO. 1	SAMPLE NO. 2
RAO	0.113	0.036
	0.114	0.030
CGH	0.116	0.029
	0.116	0.039
JTW	0.114	0.027
	0.114	0.030
LWF	0.117	0.038
	0.121	0.037
Added	0.100	0.030

Two analysts (R.A.O. and C.G.H.) suggest that directions for the removal of pectic acid should be more specific when small amounts of pectic acid and tartaric acid are present. They found it necessary to allow the mixtures to stand overnight to obtain results that checked. Changes in wording of the method as suggested have been incorporated.

LAEVO-MALIC ACID

In 1943, Hartmann (2) proposed a method for *l*-malic acid requiring a lead treatment to remove isocitric acid. This treatment makes necessary an empirical factor to correct for losses of *l*-malic acid. The writer has tried this method and was not able to get satisfactory recovery of known amounts of acid. Hartmann also said that, in the absence of isocitric acid, polarization for malic acid can be made on the isolated acid solution after removal of tartaric acid. The only available reference to isocitric acid in fruits is that of Nelson (3) who reports that five-sixths of the acids of blackberry consist of isocitric; therefore a method for *l*-malic acid in the absence of isocitric acid is proposed as follows:

METHOD FOR LAEVO-MALIC ACID

(Not applicable in presence of isocitric acid—blackberry)

Concentrate the filtrate from the tartaric acid detn (procedure above) to about 5 ml on steam bath (note 1). Cool and add NaOH (1+1) a drop at a time until alkaline to phenolphthalein, and then add just enough *N* acetic acid to discharge the phenolphthalein color. Transfer to a 25 ml volumetric flask and make to mark with H_2O. Pour the soln into a fine porosity sintered filter tube containing a mat of carbon (not 2) several mm thick. By means of pressure, force the liquid slowly through the disc, 1 or 2 ml per minute, into a 50 ml flask. If the solution is not colorless, pass through another fresh mat of carbon. Mix the solution and polarize in a 200-mm tube at room temperature, using white light. Return the solution in the polariscope tube to the remainder in the flask. Add 2.5 g of finely-powdered uranium acetate, *protect from light,* and shake in a machine for half an hour. Filter on a retentive paper in the *dark,* mix and polarize as before. Do not allow the treated solution to be exposed to light, which causes the uranium complex to become insoluble; if this is filtered off, loss of malic acid will occur. The algebraic difference between the readings in degrees Ventzke, multiplied by the factor 0.0153 (note 3) gives grams *l*-malic acid in the sample taken for the tartaric acid determination.

NOTES ON L-MALIC ACID METHOD

(Note 1)—A jet of air over surface of liquid speeds evaporation and prevents danger of loss by bumping.

(Note 2)—Merck's activated charcoal for decolorizing and Nuchar W have been used and found satisfactory.

(Note 3)—The factor is obtained by dividing the grams *l*-malic acid per degree Ventzke by 0.64 to correct for the aliquots taken in the tartaric acid determination. The grams *l*-malic acid per degree Ventzke is found by polarizing a solution of pure *l*-malic acid before and after treatment with uranium acetate. More data on this figure are being accumulated.

For the study of this method it is desirable to have a natural product that contains little or no *l*-malic acid. From results reported by Hartmann and Hillig (4) on the acid content of fruits and vegetables, more or less *l*-malic acid occurs in almost all common fruits and vegetables. However, beets are reported to have none, and raspberries to contain only very little. Therefore, three samples were prepared from these products and

sent to collaborators for analysis by the proposed method. Sample No. 1 was a beet juice (shown to be free of *l*-malic acid by analysis) with 0.71 gram *l*-malic per 100 ml acid added in the form of the lithium salt. Sample No. 2 was a commercial raspberry juice, watered somewhat in preparation. Sample No. 3 was the same as No. 2 except that 0.247 gram *l*-malic per 100 ml acid was added. Results on these three samples are given in Table 2.

TABLE 2.—*Gram l-malic acid per 100 ml*

ANALYST	SAMPLE 1, 0.71 ADDED	SAMPLE 2, NONE ADDED	SAMPLE 3, 0.247 ADDED
JTW	0.704	0.015	0.240
	0.689	0.011	0.253
LWF	0.685	0	0.230
	0.681	0	0.241
CGH	0.696	0.011	0.236
	0.696	0	0.231

Results for *l*-malic acid are in good agreement with amounts added. Analyst J.T.W. used a photographic dark room and "yellow light" when necessary to protect the solution from light.

Appreciation for collaborative work is expressed to the following members of the Food and Drug Administration: Dr. R. A. Osborn and C. G. Hatmaker of Washington, D. C., and J. T. Welch of Buffalo, N. Y.

RECOMMENDATIONS*

In "Changes in Methods of Analysis," (5) the two lines that appear at the top of page 75 were needed to regulate the addition of Rochelle salt and potassium acetate in the tartaric acid procedure as suggested by Hartmann (2). Since we now find that we obtain satisfactory results when small amounts of tartaric acid are present in the sample, without the addition of Rochelle salt and potassium acetate, these two lines are not necessary. It is therefore recommended:

(1) That the two lines beginning "dilute 20 ml . . . " at top of page 75, Vol. 34, No. 1, be deleted.

(2) That the method for tartaric acid, *Methods of Analysis*, 7th Ed. **20.35–20.36**, as redrafted be adopted as official.

(3) That the first action method for *l*-malic acid as given in the *Methods of Analysis*, 7th Ed., **20.39, 20.40, 20.41**, be retained and the procedure as presented in this year's report for *l*-malic acid (not applicable in presence of isocitric acid—blackberry) be adopted, first action.

* For report of Subcommittee D and action of the Association, see *This Journal*, 36, 63 (1953).

(4) That the study of methods for the determination of fruit acids be continued.

REFERENCES

(1) FERRIS, L. W., *This Journal*, **35**, 661 (1952).
(2) HARTMANN, B. G., *ibid.*, **26**, 459 (1943).
(3) NELSON, E. K., *J. Am. Chem. Soc.*, **47**, 568 (1925).
(4) HARTMANN, B. G., and HILLIG, F., *ibid.*, **17**, 527–529 (1934).
(5) *This Journal*, **34**, 75 (1951).

REPORT ON FROZEN FRUITS

THE ESTIMATION OF FRUIT, SUGAR, AND WATER CONTENT

By H. O. FALLSCHEER (Seattle, Wash.), *Associate Referee*, and R. A. OSBORN (Washington, D. C.), *Referee*. (Food and Drug Administration, Federal Security Agency)

A general procedure for estimating fruit and sugar content of preserves and jams has been described by Sale (1). The use of this procedure in the examination of frozen fruits for fruit and sugar content was discussed by Osborn (2). It was pointed out that it would be desirable to have a simple, rapid, and accurate alternate procedure for the determination of fruit, sugar, and water in frozen fruit, based on carefully controlled drained weight determinations coupled with the determination of soluble solids by the refractometer. This report contains data obtained by both procedures.

During the fruit packing season of 1951, in conjunction with studies on fill of container, a number of authentic packs of frozen fruits in 12 and 16 ounce consumer size packages were prepared. Fruits were representative of those grown and packed in Oregon, Washington, and California.

The following types of packs were prepared at commercial packing houses: "A", commercially washed, drained, and sorted fruits, without addition of a packing medium. "C"; commercial packs representing the product in commercial production (where feasible the actual weights of fruit and packing medium entering each individual package were determined by weighing to 0.01 oz.); "B", samples of the syrup or sugar used as packing media in the preparation of the commercial packs. "D," "E," etc. were prepared with variations in the ratio of fruit to packing media such as the concentration of the syrup, or in the ratio of fruit to sugar with fruit and packing medium weighed to 0.01 oz.

Sufficient units of each of the packs were prepared to permit collaborative examinations by Food and Drug Administration analysts located in Seattle, Portland, San Francisco, and Washington, D. C. Chemical analyses were made of the fruit alone, and on certain of the packs of fruit containing accurately weighed proportions of fruit and packing media. The methods of analysis are described in *Official Methods of Analysis* of the A.O.A.C., 7th Ed., Chapter 20 (1950).

Four packages of each sample were composited for chemical analysis after pulping in a Waring Blendor. The analytical data obtained are given in Table 1.

The amounts of insoluble solids, ash, K_2O, P_2O_5, and acidity in the "A" subdivisions of straight fruit are in good agreement with the average values for those constituents reported by Sale, and with unpublished data in the files of the Food and Drug Administration. These average values may be used to calculate the put-in proportion of fruit in frozen fruit mixtures as well as fruit content of preserves and jams and will give a close approximation of the actual fruit content of the packs.

The data in the last vertical column of Table 1, "Average Calculated Per Cent Fruit," were obtained by averaging five individually calculated values based on the relative amounts of insoluble solids, ash, K_2O, P_2O_5, and acidity. The values so obtained are in good agreement with those reported in the column "Ratio Fruit to Packing Media."

RAPID ALTERNATE PROCEDURE FOR CALCULATION OF FRUIT CONTENT IN FRUIT AND SUGAR MIXTURES

Where the frozen fruit pack is a mixture of fruit and dry sugar, the soluble solids as determined by refractometer give a very simple and quite accurate means of determining the ratio of fruit to sugar. Though not all sugar solids, the soluble solids of the fruit and the solids of the added sugar are additive on the refractometer. Thus in a 100-pound mix of 4+1 strawberries and sugar, with strawberries of 8.0 per cent soluble solids, the 80 pounds of strawberries contribute 6.4 pounds of soluble solids. This adds to the 20 pounds of sugar for a total of 26.4% soluble solids in the mixture. The equation for determining the per cent of fruit X in a fruit-sugar pack is:

$$100M = FX + (100 - X)100, \quad \text{or} \quad X = \frac{(100 - M)100}{100 - F}$$

where M is the soluble solids of the fruit sugar mixture and F is the soluble solids of the fruit.

After pulping in a Waring Blendor, each collaborator made soluble solids determinations, corrected to 20°C., on 3 to 6 individual packages of each of the subdivisions. The soluble solids obtained and the calculation of the percent fruit in the mixtures appear in Table 2. Calculations of fruit content using actual soluble solids of ingoing fruit (Table 1) are in good agreement with actual values. Use was made of the average per cent of soluble solids of authentic fruits (1) to obtain the figures in the last vertical column of Table 2. These are in good agreement with the actual fruit content of the packs.

CALCULATION OF SYRUP STRENGTH OF FRUIT AND SYRUP MIXTURES

Syrup strength of syrup-packed fruit may be calculated from soluble solids after first obtaining an estimate of the fruit content (by means of chemical analysis or from drained weight data). After the ratio of fruit to packing medium is found ($W:P$) the equation for syrup strength, Sy, becomes:

$$100M = PSy + WF, \quad \text{or} \quad Sy = \frac{100M - WF}{P},$$

where M is soluble solids of the fruit syrup mixture and F the soluble solids of the fruit. For example, let us assume that we have a ratio of 75 parts by weight of fruit

TABLE 1.—*Frozen fruit composition*
1951 Season

SAMPLE NO.	PACKING MEDIUM	RATIO FRUIT TO PKG. MED.	SOL. SOLIDS (PER CENT)	INSOL. SOLIDS (PER CENT)	ASH (PER CENT)	K_2O Mg/100g	P_2O_5 Mg/100g	K_2O % OF ASH	P_2O_5 % OF ASH	ACIDITY AS ANHYD. CITRIC	TOTALS SUGARS AS INVERT PER CENT	pH	AVERAGE CALC. PER CENT FRUIT
					SLICED STRAWBERRIES								
78-381 K (Marshall)													
A	None		9.8	2.75	.38	212	29.3	55.5	7.7	.83	7.00	3.52	
G	Sugar	80:20	28.3	2.19	.31	167	23.6	54.6	7.7	.65	25.2		79.5
H	59.6° Syrup	75:25	23.3	2.08	.30	158	24.4	53.6	8.3	.62	21.3	3.57	77.1
78-383 K (Marshall-Robinson)													
A	None		8.2	1.68	.31	161	20.9	51.8	6.7	.60	6.38		
H	59.1° Syrup	75:25	21.1	1.25	.23	118	17.2	50.9	7.4	.45	20.4		75.9
L	48.8° Syrup	75:25	18.5	1.23	.23	117	15.8	50.6	6.8	.45	17.7		74.2
M	59° Syrup	67:33	25.7	1.02	.20	101	14.4	49.8	7.1	.39	25.0	3.52	64.5
78-382 K (Marshall)													
A	None		8.1	1.99	.37	191	33.8	51.6	9.1	.66	6.05		
G	Sugar	80:20	27.0	1.64	.29	153	25.1	52.0	8.5	.55	25.8		79.4
34-994 K (Shasta-Sierra)													
A	None		7.9	1.60	.40	198	47.7	49.5	11.9	.83	5.03	3.42	
D	Sugar	80:20	25.5	1.31	.33	161	37.2	48.3	11.2	.78	24.1		81.1
37-491 K (Marshall)													
A	None		10.4	2.05	.38	197	37.0	51.2	9.6	.93	7.36	3.45	
G	Sugar	80:20	28.0	1.59	.33	163	30.3	49.3	9.2	.81	25.7		83.0
H	60.5° Syrup	75:25	23.3	1.52	.30	148	28.5	49.5	9.5	.72	20.0		76.3
M	60.5° Syrup	62.5:37.5	29.0	1.15	.25	125	24.8	49.1	9.8	.63	27.0		64.1
37-492 K (Marshall)													
A	None		11.5	2.09	.41	214	35.0	52.5	8.6	.93	8.31	3.35	
G	Sugar	80:20	28.4	1.65	.33	176	30.6	53.2	9.2	.75	26.0		82.0
H	60.5° Syrup	75:25	23.5	1.48	.31	162	29.0	52.1	9.3	.70	21.2		76.2
37-494 K (Marshall)													
A	None		11.3	2.19	.38	190	52.8	50.7	14.1	.86	8.02	3.35	
G	Sugar	80:20	29.4	1.74	.31	157	40.4	50.0	12.9	.70	26.5		80.7
H	59.9° Syrup	75:25	22.6	1.90	.27	136	37.1	51.1	13.9	.62	20.3		74.3
M	59.9° Syrup	62.5:37.5	29.5	1.33	.24	121	28.9	50.6	12.1	.53	28.0		61.2
					RED RASPBERRIES								
78-389 K (New Washington)													
A	None		14.3	6.67	.40	198	42.2	49.6	10.6	1.34	10.49	3.60	
C	55° Syrup	68:32	28.2	3.84	.27	130	23.4	48.3	8.7	1.08	25.6		65.4
H	55° Syrup	70:30	27.1	4.52	.28	142	29.2	49.9	10.3	.98	25.2		70.6
78-390 K (New Washington)													
A	None		11.8	5.20	.33	163	31.5	46.9	9.0	1.19	8.66	3.30	
C	49.7° Syrup	68:32	24.2	3.23	.27	127	27.0	47.7	10.2	.94	22.3		77.0
H	49.7° Syrup*	70:30	24.1	3.77	.27	124	23.8	46.8	9.0	.92	22.0		76.4
78-391 K (New Washington-Cuthbert)													
A	None		12.3	5.36	.38	188	36.3	49.2	9.5	1.48	9.08		
H	50.85° Syrup	70:30	24.7	3.61	.26	124	25.9	48.4	10.1	1.01	22.7		68.0
78-396 K (New Washington)													
A	None		13.5	5.36	.40	198	36.4	49.9	9.2	1.67	9.61	3.00	
H	48.8° Syrup	70:30	23.8	4.11	.27	127	25.8	46.7	9.5	1.05	21.8	3.05	67.9
					BLACK RASPBERRIES								
78-392 K A	None		14.5	8.96	.44	228	35.9	52.2	8.2	.63	10.58	3.92	
K	50.8° Syrup	75:25	26.4	7.29	.37	191	33.4	51.6	9.0	.46	22.2		83.2

Table 1—(*continued*)

Sample No.		Packing Medium	Ratio Fruit to Pkg. Med.	Sol. Solids (per cent)	Insol. Solids (per cent)	Ash (per cent)	K_2O Mg/100g	P_2O_5 Mg/100g	K_2O % of Ash	P_2O_5 % of Ash	Acidity as Anhyd. Citric	Total Sugars as Invert per cent	pH	Average Calc. per cent Fruit
Blackberries														
78-393 K (Pacific)														
	A	None		12.4	3.42	.36	170	37.1	46.8	10.2	1.64	8.44	3.1	
	K	50.8° Syrup	75:25	22.2	2.47	.29	132	28.2	46.2	9.9	1.25	19.7		76.2
78-399 K (Evergreen)														
	A	None		11.4	6.56	.50	243	44.8	49.0	9.0	1.21	7.29	3.32	
	D	Sugar	80:20	30.3	5.00	.39	190	34.5	48.5	8.8	.90	26.6		77.0
	H	50.3° Syrup	75:25	21.6	5.49	.36	168	29.6	47.3	8.3	.88	18.4		72.6
37-493 K (Himalaya)														
	A	None		14.2	6.87	.46	206	60.8	45.0	13.3	1.10	9.35	3.18	
	H	50° Syrup	75:25	21.5	4.93	.31	135	42.3	43.1	13.5	.79	17.6		69.4
	L	60° Syrup	75:25	25.3	4.92	.33	142	43.2	43.6	13.3	.83	20.9		71.7
Loganberries														
78-395 K	A	None		10.2	4.72	.37	180	27.5	49.3	7.5	1.95	6.38	2.98	
	K	59.9° Syrup	70:30	23.6	3.55	.27	132	17.9	49.4	6.7	1.54	20.4	3.00	73.2
Boysenberries														
78-394 K	A	None		11.7	3.79	.36	186	31.3	51.4	8.6	1.02	8.60	3.33	
	K	50.5° Syrup	75:25	21.0	2.92	.28	134	21.9	48.2	7.9	.80	18.8		74.8
R.S.P. Cherries														
78-397 K (Montmorency)														
	A	None		17.1	1.55	.55	304	51.2	55.7	9.4	1.01	11.35		
	H	61.2° Syrup	75:25	29.5	1.05	.41	226	36.8	55.3	9.0	.77	25.0	3.55	73.0
78-398 K (Montmorency)														
	A	None		15.5	1.42	.39	220	35.5	57.1	9.2	.98	10.33	3.38	
	D	Sugar	83:17	30.6	1.16	.32	183	29.9	57.2	9.3	.84	26.4	3.35	83.6
	H	60.4° Syrup	75:25	26.6	1.00	.29	164	26.5	57.3	9.3	.69	23.1	3.38	72.8
	M	60.4° Syrup	67:33	31.4	.92	.27	146	24.1	54.9	9.1	.61	28.3		65.9
Sliced Peaches														
34-995 K (Early Elberta)														
	A	None		9.7	.82	.35	210	38.7	60.0	11.1	.50	7.61	3.80	
	C	49.3° Syrup	74:26	20.1	.66	.31	158	29.9	51.8	9.6	.37	19.2		78.8
34-997 K (Kim Elberta)														
	A	None		10.4	.84	.31	169	38.5	54.5	12.4	.39	8.38	3.79	
	C	48.4° Syrup	69:31	22.9	.54	.22	105	21.6	49.5	9.7	.33	22.8		67.6
34-998 K (Fay Elberta)														
	A	None		9.4	1.06	.36	212	44.1	59.0	12.2	.42	7.38	3.98	
	C	52.2° Syrup	69:31	23.7	.55	.29	150	29.6	51.7	10.2	.30	23.0		68.4
34-999 K (Rio Oso Gem)														
	A			10.7	.83	.35	201	40.9	57.5	11.7	.64	8.17	3.54	
	C	49.6° Syrup	63:37	24.3	.50	.22	123	27.8	55.0	12.4	.42	23.3	3.54	63.7
Tilton Apricots														
34-996 K	A	None		13.3	1.36	.79	469	69.5	59.8	8.8	.85	8.98	4.01	
	D	47.2° Syrup	66:34	25.2	.87	.53	320	50.6	60.8	9.6	.62	22.2	4.00	69.0

* Syrup 70% Sucrose, 30% Dextrose—All other syrups are 100% sucrose.

TABLE 2.—*Fruit content of fruit-sugar packs calculated from soluble solids (S.S.)*

NUMBER	FRUIT	SUB. NO.	AV. SOL. SOLIDS (PER CENT)	FRUIT SUGAR RATIO (PUT-IN)	CALCULATED FRUIT CONTENT	
					USING S.S. OF PUT-IN FRUIT	USING AV. S.S. OF AUTHENTICS
78-381 K	Strawberries	G	27.9 (RAO)	80:20	79.9	78.5
			28.3 (HOF)		79.5	78.1
78-382 K	Strawberries	G	26.6 (RAO)	80:20	79.9	80.0
			27.0 (HOF)		79.4	79.5
37-491 K	Strawberries	G	28.0 (RAO)	80:20	80.4	78.4
			28.0 (TES)		80.4	78.4
37-492 K	Strawberries	G	27.8 (RAO)	80:20	81.6	78.6
			28.4 (TES)		80.9	
37-494 K	Strawberries	G	29.4 (TES)	80:20	79.6	76.9
34-994 K	Strawberries	D	25.5 (RAO)	80:20	80.9	81.2
			25.5 (HOF)		80.9	81.2
37-493 K	Blackberries	G	30.2 (RAO)	80:20	81.4	79.5
			30.6 (TES)		80.9	79.0
78-399 K	Blackberries	D	30.0 (RAO)	80:20	79.0	79.7
			30.3 (TES)		78.7	79.4
			30.3 (HOF)		78.7	79.4
78-397 K	R.S.P. Cherries	D	31.0 (RAO)	83.3:16.7	83.2	81.8
			29.1 (TES)		85.5	84.0
78-398 K	R.S.P. Cherries	D	29.7 (RAO)	83.3:16.7	83.2	83.3
			30.6 (HOF)		82.1	82.2

to 25 parts by weight of syrup, with $M = 21.1$ and $F = 8.2$:

$$Sy = \frac{(100 \times 21.1) - (75 \times 8.2)}{25} = 59.8.$$

Representative calculations for the various fruits appear in Table 3. It will be observed that the calculated syrup strengths are in good agreement with the Brix of the put-in syrups.

DRAINED WEIGHT

When a package of frozen fruit is allowed to thaw, and then drained, the weight of drained fruit should be related to the put-in weight of the fruit ingredient. There are factors which undoubtedly influence the

TABLE 3.—*Syrup strength calculated from soluble solids*
(Ratio fruit to packing medium known)

NUMBER	FRUIT	SUB. NO.	RATIO FRUIT TO PKG. MED.	PUT-IN SYRUP STRENGTH BRIX	SOLUBLE SOLIDS		CALCULATED SYRUP STRENGTH BRIX
					FRUIT %	MIX %	
78-382 K	Strawberries	H	75:25	60.4	8.2	21.1	59.8
		L	75:25	48.8	8.2	18.5	49.4
		M	66.7:33.3	59.0	8.2	25.7	60.8
78-396 K	Raspberries	H	70:30	48.8	13.45	23.75	47.8
78-393 K	Blackberries	H	75:25	50.8	12.4	22.2	51.6
78-398 K	R.S.P. Cherries	H	75:25	60.4	15.5	26.6	59.9
		M	66.7:33.3	60.4	15.5	31.4	63.2
34-999 K	Peaches	C	63:67	49.6	10.7	24.3	47.5
34-996 K	Apricots	C	66:34	47.2	13.3	25.2	48.3

drained weight of a given weight of fruit, such as maturity, variety, and temperature and time of draining. Over-ripe and soft fruits are more difficult to drain than are firm fruits. Some difficulty was experienced in obtaining satisfactory collaborative results for drained weights with frozen strawberries, and red raspberries. The writers have found that approximately $\frac{2}{3}$ of the put-in weight of strawberries remain on the screen, while $\frac{3}{4}$ or more of the put-in weight of red raspberries is retained. Blackberries are a firm fruit which should give reproducible drained weights. We have found that from 85 to 90 per cent of the put-in weights of this fruit remain on the screen after draining. Table 4 contains drained weight ratios, and soluble solids for the mixtures, of a number of packs of freestone peaches, apricots, and red sour pitted cherries. Portions of most of these packs were examined in three Food and Drug Administration laboratories. Each of the calculated ratios of drained weight to put-in weight of fruit is the average from the examination of 3 to 6 individual boxes of a given subdivision. The procedure for the drained weight determinations follows:

PROCEDURE FOR DRAINED WEIGHT OF FROZEN FRUITS

Determine net weight of sample by subtracting tare weight from gross weight. Remove frozen sample from container and place in a pliable bag (Cry-O-Vac, pliofilm, etc.) of convenient size. Close bag with clamp or by tying and immerse in water maintained at 20°C. ±1°C. Allow sample to reach a temperature of 20°C. (approximately 2 hours for a 1 lb. sample). Check temperature of sample by removing from water, opening bag, and inserting thermometer in the center of the sample. (Sample should be returned to water bath if contents are below 19°C.)

	NO.	MEDIUM	TO PKG. MED.	DIV. FOOD	SF	SE	AV.	DIV. FOOD	SF	SE	AV.
				PEACHES							
34-995 K	C	49.3° Syrup	74:26	19.3	18.5	18.4	18.9	70	70	72	71
	D	49.3° Syrup	65:35	22.3	21.0	21.9	22.1	71	70	71	71
	E	49.3° Syrup	65:35	—	22.6	22.3	22.3	—	70	70	70
	L	40.3° Syrup	65:35	19.4	20.5	—	19.4	72	69	—	71
34-997 K	C	48.4° Syrup	69:31	21.1	21.6	21.6	21.4	76	79	78	78
	D	48.4° Syrup	69:31	22.7	21.7	22.2	22.5	78	82	85	81
	E	48.4° Syrup	66:34	22.9	22.6	22.7	22.8	72	79	80	77
	H	48.4° Syrup	72:28	19.2	20.2	20.9	20.1	75	79	81	78
	G	48.4° Syrup	75:25	18.8	19.1	—	18.8	76	80	—	78
34-998 K	C	52.2° Syrup	69:31	23.7	22.0	21.4	22.4	81	82	83	82
	D		69:31	22.4	22.0	21.4	21.9	77	79	82	80
	E		65:35	22.8	23.5	23.2	23.2	75	83	83	81
	H		75:25	19.8	19.6	19.2	19.5	78	80	83	80
34-999 K	C	49.6° Syrup	65:35	23.3	24.1	23.4	23.6	86	86	88	87
	D		70:30	21.8	22.4	21.4	21.9	84	84	88	86
	E		65:35	23.6	23.7	23.2	23.5	84	86	91	87
	H		75:25	20.0	20.0	19.0	19.7	85	86	88	86
							(Av.)	78	79	81	79
				APRICOTS							
34-996 K	D	47° Syrup	66:34	25.0	24.5	24.1	24.5	77	83	82	81
	G		71:29	23.3	22.5	21.7	22.5	80	82	85	82
	H		61:39	26.3	25.5	24.8	25.5	82	86	87	85
							(Av.)	79	84	85	83
				RED SOUR PITTED CHERRIES							
					(PORT.)				(PORT.)		
78-397 K	D	Sugar	83:17	31.0	29.1	28.5	29.5	81	81	81	81
78-398 K	D	Sugar	83:17	29.7	—	29.3	29.5	80	87	88	84
78-398 K	H	60.4° Syrup	75:25	26.5	26.7	26.8	26.7	80	88	89	86
78-398 K	K	60.4° Syrup	80:20	24.5		24.6	24.6	82	—	85	83
78-397 K	L	61.2° Syrup	80:20	26.6		25.3	26.0	76	—	84	80
78-398 K	M	60.4° Syrup	67:33	30.4				86	—	—	—
							(Av.)	81	86	87	84

Tilt the opened container so as to distribute the contents evenly over the meshes of a circular sieve which has been previously weighed. The diameter of the sieve is 8 inches if the quantity of contents of the container is less than 3 pounds, and 12 inches if such quantity is 3 pounds or more. The bottom of the sieve is woven-wire cloth which complies with the specifications for such cloth set forth under "2380 Micron (No. 8)" in Table I of "Standard Specifications for Sieves," published March 1, 1940, in L.C. 584 of the U. S. Department of Commerce, National Bureau of Standards. Without shifting the material on the sieve so incline the sieve as to facilitate drainage. Two minutes from the time drainage begins, weigh the sieve and drained fruit. The weight so found, less the weight of the sieve, shall be considered to be the total weight of drained fruit.

CONCLUSIONS AND RECOMMENDATION

Data obtained on authentic packs of frozen fruits show that the ratio of fruit to packing medium in commercial frozen fruits may be calculated using values for ash, K_2O, P_2O_5, acidity, and water-insoluble solids, and similar average values of the corresponding authentic fruits reported in the literature. Where the packing medium is dry sugar, a soluble solids reading on the refractometer will give a rapid and quite accurate means for calculating the fruit content. After the fruit to packing medium ratio for syrup-packed fruit has been calculated the soluble solids figure gives a convenient means of calculating syrup strength. Drained weights of frozen fruits show promise as a rapid means of calculating fruit content.

It is recommended*—

That the frozen fruit work be continued with particular reference to the relation of drained weight to put-in weight of fruit.

ACKNOWLEDGMENT

We gratefully acknowledge the assistance of the following Food and Drug members in preparing the authentic packs and performing much of the analytical work: W. W. Wallace, Seattle; T. E. Strange and R. Edge, Portland; J. W. Cook, P. A. Mills, A. G. Buell, A. D. Davison, and Herman J. Meuron, San Francisco; and C. G. Hatmaker, Washington, D. C.

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The contributed paper entitled "Comparison of Results of Analysis for Potassium in Jams and Jellies by the Platinum Chloride and Flame Photometer Methods," and the paper entitled "Comparison of Analysis for Phosphorus in Jams and Jellies by Ammonium Phosphomolybdate Volumetric and Molybdenum Blue Colorimetric Procedures" will appear in a later number of *This Journal*.

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 63 (1953).

REPORT ON MEAT AND MEAT PRODUCTS

By R. M. MEHURIN (Meat Inspection Division, Bureau of Animal Industry, U. S. Department of Agriculture, Washington, D. C.), *Referee*

The two air drying methods recommended by the Associate Referee for determining moisture in meat products are substantially the same as those appearing in the Sixth Edition of *Methods of Analysis* under the heading "Added water in sausage—tentative." After the deletion by the Association of this tentative air oven drying procedure and consequent exclusive use of the more difficult official vacuum oven method, it soon became apparent that the latter method was not entirely reliable for determining moisture in very fat meat products. The comprehensive data submitted by the Associate Referee, however, demonstrate that the air oven procedure is reasonably accurate and reproducible for moisture in very fat meat products as well as for other kinds of meat products.

No report has been received from the Associate Referee on horse meat in ground meat. Some promising results have been secured in the meat inspection laboratory with a rapid method for the identification of horse meat which depends on the spectrophotometric determination of linolenic acid in the fat of the suspected meat after conjugation of the acid by heating the fat with glycerol and potassium hydroxide. It is, like the hexabromide test, applicable to cooked meat, and the test appears dependable in the presence of varying percentages of fat from common food animals as well as from deer, bear and whale.

No report was received from the Associate Referee on creatin in meat products.

It is recommended*—

(1) That methods submitted by the Associate Referee for moisture in meat products be made official, first action, and that the method for fat be modified as recommended and subjected to collaborative study.

(2) That the method for starch be subjected to further collaborative study.

(3) That work be continued on methods for the chemical and serological detection of horse meat in ground meat.

(4) That work be continued on the determination of creatin in meat products.

* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 57 (1953).

REPORT ON MOISTURE DETERMINATION IN MEAT PRODUCTS

By E. S. WINDHAM (Army Medical Service Graduate School, Washington 12, D. C.), *Associate Referee*

The suitability of the present moisture methods (*Methods of Analysis*, 7th Ed., **23.2**, referring to **22.3** and **22.7**) for use on all meat products has been questioned. Therefore, it was considered advisable to restudy moisture determination in meats.

Methods **22.3, 22.7** (*Methods of Analysis*, Seventh Edition) and the commonly used overnight drying procedure (Sixth Edition, Tentative Method **28.3**) were compared. Method **22.3** (vacuum at 95–100°) was tried both with the dishes closed and slightly open. Two sample size ranges were tried with the overnight drying at 101°–102°C. One sample size was to give about 2 g dry material. The other was about 8 g for sausages (as in Sixth Edition **28.3**) and about 10 g for meat-containing canned products, as had been specified in several military specifications for these products. All determinations were conducted in duplicate in aluminum dishes with close-fitting covers.

Six samples including pork sausage, frankfurters, and army field rations were run by the procedure using room-temperature vacuum drying over sulfuric acid (**22.7**). In most cases the results, which required at least a week to obtain, were very erratic and averaged significantly lower on each sample than those by the oven drying procedures. This method was found far too slow for practical operation and was not further studied.

Comparison of results on frankfurters, fresh pork sausage, and army field rations by the four heat-drying procedures referred to above indicated that the lids must be slightly opened for proper drying under vacuum. Half the samples dried with closed dishes gave trouble varying from slight greasiness outside the dish to exploding, foaming out, and failure to dry at all. Eight out of 42 determinations by the vacuum oven method, with lids of dishes partly open, were partially or wholly unsatisfactory for the same reasons. Small or large samples seemed equally satisfactory for drying frankfurters and canned meat-containing products by the overnight drying procedure. Small samples, i.e. giving ca 2 g dry material, appear to be superior for fresh pork sausage.

Since an overnight drying procedure is more convenient for many laboratories, comparisons were made of vacuum drying at 98–100°C. for 6 and 16 hours. Separate samples of frankfurters, pork sausage, beef and gravy, and pork and gravy were dried for 6 and 16 hours, under vacuum, and 16 hours in an air oven. Another set of samples were dried for 6 hours one day in an air oven, weighed and calculated, then dried 6 more hours the following day for a total of 12 hours, are reweighed and calculated.

The 6 and 16 hours vacuum drying and the 16 hour air oven drying compared closely in most cases. The 12 hour air drying did not consistently agree with these three methods. The 6 hour air drying seldom produced results agreeing with the other methods; therefore, the 6 and 12 hour air drying methods were dropped from consideration. Since 6 hour vacuum drying agreed with 16 hour vacuum drying, the longer vacuum method was dropped as being unnecessary. Both 6 and 16 hour vacuum drying tended to give trouble, with possible loss of sample during drying on high-fat samples and products that can foam under vacuum.

As a result of this study, and from correspondence and discussion with prospective collaborators, the following instructions were issued to collaborators:

APPARATUS

(a) *Aluminum dishes.*—Ca 25 mm high by 50 mm diam., with close-fitting slip-over cover. (If other type dish is used, please describe.)

(b) *Air oven set at 100–102°C.*—Mechanical convection type is preferred. Please specify if gravity convection oven is used.

(c) *Vacuum oven set at 98–100°*C.—Must maintain vacuum of at least 26 inches (100 mm Hg pressure) when admitting at least two bubbles of air per second through sulfuric acid air-drying train. Silicone high vacuum grease is recommended for sealing door and vacuum connections.

(d) *Vacuum oven set at 69–71°C.*—Vacuum conditions same as above.

(e) *Air oven, mechanical convection, set at 125°C.*

SAMPLING

Weigh into each dish sufficient well-prepared sample to give ca 2–2.5 g of dry material, spreading as evenly as practicable over bottom of dish. Have dishes closed during weighing to minimize moisture loss.

DETERMINATIONS

METHOD 1

Open lids of dished very slightly (do not remove). Dry to constant weight (ca 6 hours) in vacuum oven at 98–100°C., under pressure not exceeding 100 mm Hg. During drying admit at least 2 bubbles of air per second passed through sulfuric acid. Remove from oven, close dishes, and cool in desiccator. Weigh soon after reaching room temperature.

METHOD 2

Remove lids from dishes. Dry 16–17 hours in atmospheric mechanical convection oven at 100–102°C. Remove from oven, close dishes, and cool in desiccator. Weigh soon after reaching room temperature.

METHOD 3

Open lids of dishes slightly (do not remove). Dry 16–17 hours in vacuum oven at 69–71°C. under pressure not exceeding 100 mm Hg. During drying admit at least two bubbles of air per second passed through sulfuric acid. Remove from oven, close dishes, and cool in desiccator. Weigh soon after reaching room temperature.

METHOD 4

Remove lids from dishes. Dry to constant weight, ca 2.5–3.5 hours, in atmospheric mechanical convection oven at a temperature of about 125±5°C. Remove from oven, close dishes, and cool in desiccator. Weigh soon after reaching room temperature.

NOTES

1. Triplicate determinations should be made by each method.
2. Determine moisture on as many products as are available that are normally run for moisture in your laboratory. Use regular, thorough sample preparation. Run as many samples of each product as you find time for, up to 20 of each product.

RESULTS

In the present A.O.A.C. vacuum method, "(ca 5 hrs)" was changed to "(ca 6 hrs)" since it was found that many samples were not at constant weight in 5 hours, particularly when samples were placed in the oven before it attained operating temperature, as is common practice.

The results of this study on pork sausages is given in Table 1. Similar types of cured sausage, are presented in Table 2. Other products are listed as miscellaneous in Table 3.

With pork sausage, of the 98 individual determinations reported by method 1, 21 were considered a total loss and at least 30 others had fat on the lid or outside the dish in sufficient quantity to be reported. Collaborators 1, 2, and 7 reported some dishes had possible loss of fat without listing which of their reported results were involved, if any. Consequently, these results were not listed in Table 1.

The other three methods proved satisfactory. The average range for methods 2, 3, and 4 respectively were 0.456, 0.520, and 0.499 per cent. For the samples on which methods 2 and 4 were compared, method 2 averaged 0.14 per cent lower than did method 4. Similarly, method 2 averaged 0.13 per cent lower than method 3. Method 3 averaged 0.36 per cent lower than method 4. None of these deviations are considered significant considering the nature of pork sausage.

The four methods studied gave closely comparable results on cured sausage-type products (Table 2) except that method 3 tended to give lower results. For the samples comparing methods 1 and 3, method 3 averaged 0.63 per cent lower. Similarly calculated, method 2 averaged 0.16 per cent lower, and method 4 averaged the same as method 1. This may be due to the tendency of method 1 to have slight spattering of the fat on many determinations. Average ranges for all determinations were: method 1, 0.381 per cent; method 2, 0.328 per cent; method 3, 0.381 per cent; and method 4, 0.273 per cent. For sausages, it would seem that methods 2 and 4 were equivalent to method 1, with method 3 being inferior when considering method 1 as a check method, since the results average so much lower.

Too few samples of ground beef, canned luncheon meat, ham and bacon (Table 3) were analyzed to justify a comparison of the methods. Nine

TABLE 1.—*Meat product moisture method comparison—pork sausage*
(Average and range of triplicates)

COLLABORATOR	METHOD 2		METHOD 3		METHOD 4	
	MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE
	per cent		*per cent*		*per cent*	
2	41.30	0.57			41.54	0.73
2	53.60	0.32			53.42	0.52
2	36.37	0.24			36.92	0.15
3	50.12[1]	0.42			50.49[1]	0.23
3	42.29[1]	0.41			42.68[1]	0.44
4	41.29	0.56	41.36	0.37	41.53[1]	0.46
5	44.37	0.47	44.05	0.49	44.22	0.13
7	43.30	0.50	42.80	0.50	43.80	0.70
7	44.80	0.60	44.70	0.80	45.20	0.50
8	35.73	0.33	35.60	0.65	35.88	0.72
8	37.42	0.57	37.67	0.24	37.59	0.58
8	36.32	1.07	35.98	0.95	36.06	0.92
8	32.77	0.61	32.61	0.27	32.75	0.19
8	37.01	1.01	36.39	0.53	36.73	0.29
8	33.30	0.65	33.71	0.43	33.56	0.06
11	44.92	0.45			45.18	0.60
11	48.55	0.74			47.82	0.36
11	40.53	0.33			40.07	0.14
11	44.24	0.28			44.24	0.47
11	44.66	0.32			44.27	0.18
11	43.07	0.39	42.85	0.19	42.91	0.17
11	37.56	0.41	38.35	0.11	38.22[2]	0.14
11	40.29	0.34	40.69	0.22	40.94	0.66
11	48.52	0.34			48.74	0.96
12	44.47	0.28	44.50	0.46		
12	43.58	0.28	43.37	0.37		
12	38.96	0.31	42.24	1.26		
12	43.73	0.15	43.54	0.19		
12	43.27	0.27	43.18	0.22		
15			46.11	0.18	45.41[1]	0.50
15			45.61	0.42	45.58[1]	0.97
15			41.40	1.48	44.76[1]	0.42
15			40.95	0.75	41.26[1]	0.54
15			39.59	0.89	40.51[1]	1.39
Number of Samples	29		23		28	
Average Range	0.456		0.520		0.499	

[1] Gravity convection oven used.
[2] Duplicate results. Not included in average range calculations.

TABLE 2.—*Meat product moisture method comparison—cured sausage type products*
(Range and averages of triplicates)

PRODUCT AND COLLABORATOR		METHOD 1		METHOD 2		METHOD 3		METHOD 4	
		MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE
		per cent		*per cent*		*per cent*		*per cent*	
Wieners	2			46.96	0.25			46.83	0.15
Garlic Saus.	2			47.85	0.19			48.49	0.23
Bologna	2			52.01	0.47			52.11	0.59
Liver Saus.	2			53.27	0.72			53.75	0.34
Potted Meat	2			67.75	0.19			67.77	0.13
Frankfurter	2			56.59	0.34			56.66	0.12
Leona	2			50.53	1.07			51.23	0.14
Mixed Saus.	2			51.41	0.38			51.96	0.11
Mixed Saus.	2			57.76	0.04			57.82	0.23
Wieners	2			47.85	0.96			47.04	0.12
Mortadella	2			58.36	0.11			58.58	0.18
Frankfurter	2			58.97	0.38			59.02	0.24
Wieners	2			57.04	0.27			57.21	0.41
Bologna	2			52.67	0.55			53.04	0.10
Bologna	2			52.40	0.37			52.25	0.32
Minced Saus.	2			50.14	0.15			50.07	0.42
Frankfurter	2			52.71	0.36			52.74	0.20
Bologna	2			58.58	0.10			58.45	0.30
Bologna	2			57.20	0.36			57.83	0.58
Wieners	2			50.73	0.29			51.17	0.28
Frankfurter	2	53.76	0.98			54.64	0.22	53.08	[3]
Minced Saus.	2	60.80	0.15			60.40	1.29	59.57	[3]
Frankfurter	2	55.15	0.24			53.87	3.14	54.50	[3]
Minced Saus.	2	58.35	0.40			58.43	0.62	58.30	[3]
Frankfurter	3	56.04	0.17	55.83[1]	0.42			55.83[1]	0.14
Bologna	3	56.10	0.12	55.70[1]	0.17			55.95[1]	0.09
Bolo. & Franks	3	53.83	0.25	54.11[1]	0.09			54.18[1]	0.14
Bologna	3	50.38	0.73	49.99[1]	0.16			50.22[1]	1.08
Bologna	3	53.18	0.08	53.06[1]	0.18			53.35[1]	0.07
Frankfurter	3	65.59	0.15	65.32[1]	0.14			65.60[1]	0.23
Cervelat	3	57.62	0.69	57.52[1]	0.36			57.72[1]	0.37
Cervelat	3	52.20	0.16	52.22[1]	0.26			52.11[1]	0.68
Frankfurter	3	56.97	0.53	56.37[1]	1.72			56.74[1]	0.43
Frankfurter	3	55.49	0.25	55.30[1]	0.09			55.38[1]	0.22
Bologna	4	61.72	0.39	61.61	0.21	61.02	0.31	61.37[1]	0.04
Bologna	4	57.88	0.08	57.83	0.08	57.57	0.15	58.06[1]	0.11
Frankfurter	4	56.30	0.12	56.14	0.05			56.30[1]	0.09
Frankfurter	5	55.14	0.19	55.42	0.08	54.42	0.22	55.46	0.12
Bologna	5	53.57	0.43	53.45	0.11	52.58	0.32	53.47	0.31
Frankfurter	8	54.64[2]	0.03	54.28	0.07	54.58	0.33	54.93	0.26
Bologna	8	55.27	0.36	55.09	0.37	55.97	0.04	56.00	0.25
Bologna	8	53.67	0.14	52.42	0.60	53.57	1.17	54.34	0.31
Frankfurter	8	55.70	0.94	55.26	0.92	54.29	0.41	55.83	0.27
Frankfurter	8	54.95	0.08	54.99	0.48	54.38	0.63	55.26	0.27
Frankfurter	11	51.73	0.55	51.64	0.29			51.91	0.15
Frankfurter	11	51.75	0.76	51.91	0.41			52.26	0.16
Frankfurter	11	57.41	0.29	57.39	0.35	57.11	0.16	57.29	0.19
Frankfurter	11	51.09	0.41	50.85	0.27	50.44	0.10	50.89	0.14
Salami	11	59.29	0.30	58.84	0.22	58.34	0.62	58.90	0.41
Frankfurter	11	50.61	1.39	51.90	0.53	51.75	0.29	52.00	0.06
Frankfurter	11	50.44	0.38	51.05	0.05			51.43	0.18
Frankfurter	11	54.30	0.09	53.95	0.18	53.84	0.26	54.15	0.23

[1] Gravity convection oven used.
[2] Duplicate determinations. Not included in averages of range.
[3] Single determinations. Not included in averages of range.

TABLE 2—(*continued*)

PRODUCT AND COLLABORATOR		METHOD 1		METHOD 2		METHOD 3		METHOD 4	
		MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE
		per cent		*per cent*		*per cent*		*per cent*	
Salami	11	52.77	0.32	52.56	0.80	52.44	0.47	52.55	0.21
Frankfurter	11	53.20	0.22	52.87	0.27	52.63	0.29	53.06	0.29
Frankfurter	11	53.99	0.66	53.99	0.21	53.77	0.23	53.97	0.69
Frankfurter	11	53.60	0.46	53.45	0.06			53.53	0.37
Bologna	11	55.70	1.01	55.62	0.39			55.35	0.40
Bologna	11	49.91	0.36	50.02	0.05			50.12	0.52
Frankfurter	11	54.07	0.16	54.75	0.40			54.92	0.35
Frankfurter	11	57.90	0.11	57.55	0.54	57.55	0.16	57.79	0.21
Frankfurter	11	56.70	0.34	56.42	0.47	56.81	0.17	56.87	0.14
Frankfurter	11	56.62	0.19	55.88	0.37	56.43	0.26	56.39	0.41
Frankfurter	11	52.07	0.14	52.32	0.06	51.93	0.32	52.39	0.23
Salami	11	54.36	0.29	53.90	0.30	53.70	0.17	53.97	0.25
Salami	11	53.51	0.48	53.62	0.08	53.58	0.15	53.48	0.12
Salami	11	55.49	0.04	55.70	0.12			55.35	0.54
Bologna	11	53.46	0.23	52.64	0.51			53.00	0.13
Frankfurter	11	50.71	0.25	50.78	0.18			50.91	0.28
Frankfurter	11	52.42	0.32	52.38	0.35			52.48	0.41
Frankfurter	11	57.52	0.13	57.25	0.47			57.41	0.09
Frankfurter	11	51.39	0.33	51.16	0.34	50.23	0.74	51.31	0.24
Frankfurter	11	50.47	0.72	50.27	0.33	48.73	0.62	50.49	0.41
Frankfurter	11	56.06	0.06	55.83	0.03	55.77	0.11	55.98	0.20
Frankfurter	11	55.65	0.22	55.10	0.09	54.69	0.24	55.11	0.29
Frankfurter	11	52.25	0.39	51.95	0.26	51.51	0.12	52.12	0.08
Frankfurter	11	54.15	0.33	54.05	0.47	53.39	0.44	54.35	0.24
Frankfurter	11	56.98	0.38	57.09	0.24	55.86	0.39	56.98	0.45
Bologna	11	58.04	0.26	57.37	0.35	56.75	0.48	57.99	0.24
Bologna	11	56.99	0.28	56.98	0.05	55.73	0.21	57.15	0.19
Salami	11	50.91	0.27	50.26	0.06	50.18	0.27	50.51	0.30
Salami	11	55.41	0.80	54.97	0.43	54.50	0.58	54.99	0.19
Bologna	11	53.01	0.17	52.64	0.70	52.59	0.11	53.25	0.20
Bologna	11	54.36	0.31	53.92	0.25	53.81	0.27	54.32	0.17
Luncheon Meat	11	60.39	0.38	60.36	0.54	59.87	0.34	60.53	0.28
Luncheon Meat	11	58.28	0.26	57.62	0.36	57.45	0.41	57.54	0.09
Frankfurter	11	47.07	0.39	47.18	0.06	47.11	0.03	47.33	0.10
Frankfurter	11	49.46	0.18	49.56	0.09	49.35	0.29	49.73	0.13
Frankfurter	11	58.77	0.08	58.42	0.21	58.37	0.07	58.68	0.21
Frankfurter	11	47.96	0.30	48.19	0.09	47.77	0.34	48.39	0.21
Frankfurter	11	53.81	0.16	53.97	0.16	53.97	0.11	54.09	0.11
Frankfurter	11	48.12	0.10	48.26	0.13	47.81	0.17	48.31	0.21
Frankfurter	11	56.83	0.32	56.59	0.36	56.25	0.58	56.52	0.35
Frankfurter	11	54.55	0.12	54.36	0.10	54.36	0.33	54.48	0.09
Salami	12			58.28[2]	0.05	58.25	0.28		
Salami	12			57.44	0.16	57.44	0.09		
Salami	12			59.12	0.07	59.10	0.19		
Salami	12			54.02	0.13	54.02	0.14		
Salami	12			51.08	1.32	50.70	0.07		
Bologna	12			57.14[2]	0.24	56.70	0.26		
Bologna	12			55.62	0.72	55.86	0.14		
Bologna	12			55.22	0.39	55.34	0.30		
Bologna	12			62.78	0.76	62.65	0.34		
Bologna	12			55.01	0.31	54.86	0.23		
Bologna	12			58.38	0.44	58.30	0.30		
Bologna	12			53.74	0.41	53.65	0.37		
Liver Saus.	12			49.83[2]	0.21	49.76	0.08		
Liver Saus.	12			54.08	0.63	53.94	0.33		
Liver Saus.	12			48.67	0.08	48.65	0.32		
Liver Saus.	12			51.34	0.10	51.34	0.16		
Frankfurter	12			54.62	0.99	54.28	0.22		

TABLE 2—(*continued*)

PRODUCT AND COLLABORATOR		METHOD 1		METHOD 2		METHOD 3		METHOD 4	
		MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE
		per cent		*per cent*		*per cent*		*per cent*	
Frankfurter	12			50.75	0.10	50.63	0.12		
Frankfurter	12			50.73	0.29	50.55	0.12		
Frankfurter	12			52.91	1.12	53.24	0.13		
Frankfurter	12			56.98	0.08	56.93	0.10		
Frankfurter	12			56.50	0.22	56.54	0.21		
Frankfurter	12			60.36	0.12	60.42	0.15		
Frankfurter	12			53.08	0.68	53.21	0.16		
Frankfurter	12	51.19	0.78	50.76	0.06				
Frankfurter	12	54.17	2.05	53.76	0.03				
Frankfurter	12	54.50	1.10	53.77	0.12				
Salami	15	53.55	0.39			49.57	1.86	53.00[1]	0.58
Frankfurter	15	49.76	0.44			46.43	1.75	49.75[1]	0.58
Frankfurter	15	51.31	0.47			50.08	0.50	51.50[1]	0.65
Frankfurter	15	57.85	0.57			56.18	1.08	57.80[1]	0.79
Total Samples		79		113		78		93	
Average Range		0.381		0.328		0.381		0.273	

samples of canned products containing meat with vegetables were reported in Table 3. The first three methods averaged almost identically: 74.20, 74.17, 74.13 per cent respectively; while method 4 averaged 74.42 per cent. Therefore, the 3 hour drying time may be too harsh for these products, or the drying temperature of ca 125°C. may be too high.

DISCUSSION

Among the four methods studied collaboratively are found procedures suitable for moisture determination on all products. The present official methods are not wholly satisfactory. Drying over sulfuric acid at room temperature is not reproducible and is too time consuming. The 100° vacuum oven method was unreliable because of fat loss, particularly in high fat products. The 70°C. overnight vacuum method gives reliable results on all products, but limits the number of determinations that can be made due to the small capacity of most vacuum ovens. Also, overnight operation of vacuum is not feasible in many laboratories. Both the 100–102°C. overnight air oven method and the 125° short-time air oven method give reliable results and serve to meet the needs of various laboratories.

The results of collaborator No. 9 were omitted, since his figures were reported only as averages and therefore could not be further evaluated. Results of collaborator 1 and some results of collaborator 2 were omitted, as only duplicates were reported.

COMMENTS OF COLLABORATORS (CONDENSED)

E. A. Bayer: Although care was exercised in the vacuum drying (lids of weighing bottles were opened only slightly and air circulated was closely governed) there

TABLE 3.—*Meat product moisture method comparisons*

(Range and average triplicates)

PRODUCT AND COLLABORATOR		METHOD 1		METHOD 2		METHOD 3		METHOD 4	
		MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE	MOISTURE	RANGE
		per cent		*per cent*		*per cent*		*per cent*	
Hamburger	1[2]	62.21	0.02	61.83[1]	0.20			62.40[1]	0.40
Hamburger	1[2]	60.47	0.52	60.35[1]	0.20			59.93[1]	0.12
Hamburger	2[2]	58.86	0.26			59.30	0.13	58.55	0.0
Smoked Ham	3	Greasy		58.49[1]	0.27			58.85[1]	0.15
Smoked Ham	3	67.38	0.10	67.48[1]	0.05			67.42[1]	0.15
Smoked Ham	3	68.87	0.05	67.08[1]	0.10			68.89[1]	0.16
Bacon	3	27.52	0.17	26.94[1]	0.27			27.42[1]	0.23
Cooked Ham	5	63.36	0.08	63.27	0.11	63.05	0.06	63.41	0.10
Canned Lunch Meat	8	53.21	0.08	53.38	0.37	53.19	0.51	53.26	0.30
Ground Beef	7	57.8	0.1	56.7[3]	0.4	57.0	0.8	57.7	0.4
Ground Beef	7	61.1	0.4	60.3[2]	0.4	60.5[2]	0.1	60.7	0.0
Chicken & Noodles	8	70.20	0.04	69.89	0.06	70.03	0.02	70.22	0.02
Meat & Spaghetti	8	72.32	0.12	72.07	0.10	72.61	0.02	72.76	0.05
Corned Beef Hash	8	68.67	0.08	68.80	0.07	68.50	0.07	68.76	0.05
Beans with Pork	8	70.11	0.07	70.17	0.05	69.89	0.03	70.47	0.01
Beef Stew	8	82.95	0.04	83.08	0.06	82.73	0.04	83.21	0.04
Meat & Noodles	8	74.16	0.07	73.14	0.14	74.00	0.12	74.20	0.02
Lamb Stew	8	82.97	0.05	82.84	0.04	83.05	0.03	83.13	0.0
Corned Beef Hash	8	71.05	0.06	70.94	0.11	71.09	0.03	71.19	0.04
Ham & Eggs with Potatoes	8	75.38	0.03	75.58	0.05	75.27	0.03	75.81	0.02

[1] Gravity convection oven used.
[2] Duplicate determinations.
[3] Sextuplicate determinations.

was evidence that a small amount of fat escaped with the moisture. Glass weighing bottles of ca 50×40 mm were used as moisture dishes in all instances. No relation could be detected between the degree of darkening on drying and the moisture content.

F. D. Roach: Aluminum dishes, 70 mm diam., 30 mm deep, with slipover covers were used. I believe that the use of the vacuum oven is unsuitable for determination of moisture in meat products because of spattering. This spattering seems to be at a maximum in products such as pork sausage. I consider the use of the forced draft oven at 125°C. for ca 3 hours as the best method. The results are easily duplicated and it is a rapid method.

H. R. Cook: Aluminum cans ca 25×65 mm with slip-over covers were used in methods 2 and 4. Glass weighing dishes ca 30×50 mm with covers could not be used in method 1 due to considerable spattering and too great a possibility of loss of product other than moisture.

R. P. Dragoo: Dishes used were 40×50 mm glass weighing bottles. Some difficulty was experienced in heating to constant weight on all methods except method 1.

D. M. Doty and Thomas Keyahian (From very complete and comprehensive comments): Used pill boxes with close fitting covers. Suggest leaving lids off the dishes during 70°C. overnight vacuum drying. Some spattering occurred with pork sausage dried by method 1. Meat dried at 125°C. was badly discolored and had a

very strong burned odor. Suggest experimentation to establish proper sample size and drying time for each product by this method.

W. H. Dieterich: The only comment we can make is that some trouble was experienced with the vacuum oven methods.

S. W. Thompson, Jr.: In following procedure designated as method 1, a substantial loss of product other than moisture was noted.

Mr. Roach reported on the modification of the vacuum oven method which seems highly promising: remove lids from dishes and place samples in vacuum oven at 100°C. Heat one hour with rapid passage of air through oven and with vacuum no greater than 12 inches. After 1 hour, increase vacuum to 15 inches and heat to constant weight. About 5 hours is sufficient for sausages. A total of 21 samples was tested and close agreement was found with the 125°C. air oven method. The method seems to merit further study.

Mr. Hilty reported a study of methods 1, 2, and 4 with varying drying time on frankfurters, ham, bacon, and cervelat. After reaching what is considered constant weight, all three methods continued to lose weight slowly, in most cases. Some cases of increase in weight with continued heating occurred. Further study may be conducted, or a more detailed report of Mr. Hilty's findings may be reported later.

LIST OF COLLABORATORS

E. A. Boyer, Chemist in Charge, U. S. Meat Inspection Laboratory, Chicago, Illinois.

F. D. Roach, Chemist, U. S. Meat Inspection Laboratory, St. Louis, Missouri.

H. R. Cook, Chemist, U. S. Meat Inspection Laboratory, Omaha, Nebraska.

R. P. Dragoo, Chemist, U. S. Meat Inspection Laboratory, Washington, D. C.

J. W. Greer, Assoc. Chemist., U. S. Meat Inspection Laboratory, San Francisco, California.

D. M. Doty and Thomas Keyahian, American Meat Institute Foundation, Chicago, Illinois.

Flo H. Ward, Veterinary Division, Walter Reed Army Medical Center, Washington, D. C.

W. H. Dieterich, Veterinary Branch, First Army Area Medical Laboratory, New York, New York.

William H. Schiefelbein, Veterinary Branch, Third Army Area Medical Laboratory, Fort McPherson, Georgia.

S. W. Thompson, Jr., Veterinary Division, Fourth Army Area Medical Laboratory, Ft. Sam Houston, Texas.

T. O. Downing, Veterinary Division, Sixth Army Area Medical Laboratory, Seattle, Washington.

RECOMMENDATIONS*

It is recommended that the following changes be adopted, first action:

1. Change **23.2** as follows: Drop "or **22.7**." Reword as follows: **23.2**. Proceed at directed under **22.3** with lids of dishes only slightly open (not suitable for high fast products such as pork sausage), or by one of the following:

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* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 57 (1953).

(a) Dry, with lids removed, quantity of sample representing ca 2 g of dry material 16–18 hrs at 100–102° in air oven (mechanical convection preferred). Use covered Al dish at least 50 mm in diam. and not exceeding 40 mm in depth. Cool in desiccator and weigh. Report loss in weight as moisture.

(b) Dry, with lids removed, quantity of sample representing ca 2 g of dry material to constant wt (2–4 hrs depending on product) in a mechanical convection oven at ca 125°. Use covered Al dish at least 50 mm in diam. and not exceeding 40 mm in depth. Avoid excessive drying. Cover and cool in desiccator and weigh. Report loss in wt as moisture. (Note: dried sample is not satisfactory for subsequent fat detn.)

ACKNOWLEDGMENT

I wish to render my thanks for the extensive help of Mr. R. M. Mehurin, the Referee on Meat and Meat Products, in planning and evaluating this study and in obtaining collaborators.

Special thanks go to Mr. F. B. Hilty of the Omaha Meat Inspection Division Laboratory and Mr. F. D. Roach of the St. Louis Meat Inspection Laboratory for their additional work on moisture methods.

REPORT ON FAT IN MEAT PRODUCTS

By E. S. WINDHAM (Army Medical Service Graduate School, Washington 12, D. C.), *Associate Referee*

Much interest has been shown in the past few years in the substitution of petroleum ether (30–60°C. boiling range) for anhydrous ethyl ether as the solvent in the Soxhlet extraction of fat in meat products. Actually, the change in methods is now listed in the Federal Specification for pork sausage (Section 4.2.1 of PP-S-91a, Dec. 20, 1951) and in military specifications for ground beef, canned pork and gravy, and canned beef and gravy.

Petroleum ether is more satisfactory than ethyl ether in that it does not dissolve water and therefore remains anhydrous. It does not dissolve or carry over starches, sugars, or other non-fat materials into the fat flask. Water extraction of these materials prior to fat extraction is not needed. No satisfactory method has been found in this laboratory for the water extraction of meat samples for fat analysis.

EXPERIMENTAL

During the past four years numerous comparisons of ether and petroleum ether as solvents for fat extraction have been made. About two-thirds of these comparisons were carried out on pork sausage. Several products containing added starch, sugars, and milk solids were compared. The results are reported in Tables 1A and 1B. Except where noted, averages of six determinations by each method on each sample are reported. The samples were spread inside the thimble, weighed, and dried

TABLE 1.—*Comparison of ether and petroleum ether in fat determination on meat products*

A—Pork Sausage

SAMPLE	FAT, PER CENT		AVERAGE DEVIATION		SAPONIFICATION NUMBER		UNSAPONIFIABLE RESIDUE	
	ETHER	PETR. ETHER	ETHER	PETR. ETHER	ETHER	PETR. ETHER	ETHER	PETR. ETHER
189	41.48	41.39	0.25	0.25	208.1	215.6	0.50	0.46
265	40.48	40.32	0.20	0.27	214.8	214.3	0.41	0.43
282	36.96[1]	37.11	0.34	0.38	203.4	205.0	0.41	0.37
285	36.99[1]	37.41	0.10	0.33	213.3	215.8	0.63	0.48
38	45.85[1]	45.89	0.32	0.13	207.4	208.7	0.40	0.61
31	43.47	43.14	0.08	0.10	209.0	208.3	0.42	0.40
908	44.46	44.40	0.23	0.26	217.9	213.5	0.36	0.38
81	44.32	44.29	0.10	0.09	204.6	202.9	0.42	0.24
64	40.69	40.13	0.21	0.19	220.7	222.0	0.71	0.68
918	42.69	42.59	0.12	0.20	215.4	215.2	0.57	0.46
931	43.75	43.43	0.12	0.17	210.1	201.4	0.25	0.35
968	45.19	45.10	0.31	0.26	218.4	219.1	0.41	0.47
8	45.24	25.26	0.26	0.18	204.8	206.4	0.31	0.26
9	43.05[2]	42.70	0.36	0.17	204.1	200.0	0.56	0.29
821	43.29	43.25	0.23	0.18	199.7	213.3	0.34	0.50
842	44.20	44.14	0.22	0.24	206.0	206.1	0.35	0.46
844	40.29	40.13	0.24	0.23	209.6	206.3	0.29	0.32
871	42.29	42.34	0.32	0.17	209.6	213.0	0.47	0.31
872	48.34	48.54	0.23	0.20	213.2	214.3	0.38	0.37
882	43.76	43.79	0.23	0.31	208.0	208.4	0.18	0.36
887	46.58	46.80	0.30	0.48	206.1	208.5	0.48	0.33
907	44.87	44.26	0.28	0.15	210.2	205.4	0.31	0.32
145	38.52	38.49	0.18	0.31				
683	38.65	38.51	0.14	0.21	203.7	203.6	0.34	0.33
708	42.88	42.61	0.35	0.31	214.8	214.8	0.34	0.38
709	38.74	38.98	0.47	0.30	216.5	214.7	0.46	0.44
728	38.00	37.71	0.33	0.27	210.4	210.8	0.24	0.33
729	36.92	36.91	0.20	0.21	212.3	215.1	0.32	0.33
788	40.71	40.87	0.24	0.18	203.3	199.8	0.29	0.40
789	44.42	44.09	0.47	0.21	201.4	201.3	0.29	0.43
822	42.97	43.67	0.33	0.18	205.3	205.9	0.28	0.43
1	42.28	41.92	0.48	0.87	208.9	211.7	0.38	0.35
3	42.77	42.56	0.74	0.58			—	0.46
6	40.99	40.66	0.26	0.16			0.32	0.30
637	37.31	37.09	0.25	0.30	213.5	213.8	0.52	0.47
638	38.38	38.72	0.29	0.25	200.8	199.3	0.41	0.34
652	39.09[3]	39.17	0.15	0.19	213.6	212.2	0.40	0.41
653	40.00	40.31	0.12	0.16	216.7	222.6	0.53	0.57
682	39.43	39.35	0.29	0.27	205.0	207.0	0.41	0.46
685	43.79	44.24	0.43	0.27				
184	41.49	41.25	0.45	0.27	216.0	225.7	0.65	0.48
32	47.45	47.97[3]	0.36	0.44	205.0	213.8	0.54	0.56
730	39.93	39.56	0.15	0.22	211.1	211.0	0.46	0.51
230	41.53	41.77	0.16	0.09	208.5	207.9	0.52	0.39
649	41.98	41.88	0.49	0.30	206.7	205.9	0.28	0.42
513	35.43	36.30	0.36	0.26	211.1	206.7		
185	40.79	41.44	0.51	0.63	209.6	208.2		
Averages	41.78	41.78	0.28	0.26	209.5	210.1	0.41	0.41

TABLE 1—(*continued*)

B—Miscellaneous Products

PRODUCT AND SAMPLE NO.		FAT, PER CENT		AVERAGE DEVIATION		SAPONIFICATION NUMBER		UNSAPONIFIABLE RESIDUE	
		ETHER	PETR. ETHER	ETHER	PETR. ETHER	ETHER	PETR. ETHER	ETHER	PETR. ETHER
Ground Beef	16	20.30	20.29	0.14	0.21	192.1	196.1	0.59	0.54
Ground Beef	182	11.98	12.01	0.24	0.30	204.4	201.2	0.91	1.12
Ground Beef	44	30.55	30.38	0.16	0.33			0.64	0.65
Ground Beef	458	19.52	19.49	0.14	0.07	204.1	204.3	0.95	1.11
Ground Beef	S-1	15.08	14.83	0.20	0.16				
Ground Beef	S2F	14.30	14.11	0.10	0.08			0.90	0.64
Ground Beef	S-3	14.78	14.69	0.06	0.09				
Ground Beef	S-4	15.27	15.28	0.15	0.17				
Ground Beef	A-3	22.54	22.94	0.35	0.34	200.8	200.2	0.75	0.70
Ground Beef	348	12.88	12.85	0.10	0.13			0.80	0.79
Ground Beef	180	18.88	18.99	0.31	0.19	202.8	197.0	0.79	0.58
Corned Beef	1	16.81	16.79	0.01	0.06	201.3	199.5	1.07	0.98
Bologna	17	27.43[2]	27.21	0.14	0.12	203.1	204.3	0.83	0.77
Ham	3	30.52	30.62	0.16	0.35	210.6	211.1	0.58	0.51
Ham	5	21.75	21.65	0.09	0.03	214.2	214.1	0.71	0.69
Frankfurter	156	21.64	21.56	0.07	0.11				
Frankfurter[4]	Z-3	25.77[2]	25.71	0.03	0.05				
Frankfurter[4]	M-7	27.07	26.92	0.21	0.14				
Bologna[4]	M-4	24.76	24.79	0.30	0.04				
Corned Beef Hash[4]	1	27.52[2,5]	27.36	0.09	0.10	200.8	202.9	0.64	0.68
Corned Beef Hash[4]	3	17.35	17.16	0.34	0.02	200.9	196.7	0.36	0.35
Corned Beef Hash[4]	4	27.01	27.14	0.14	0.06	199.2	199.2	0.80	0.65
Beef and Gravy[4]	Z-4	14.18[3]	14.20[3]	0.06	0.04				
Pork and Gravy[4]	Z-2	36.34[3]	36.32[3]	0.20	0.16				
Frankfurter[4]	M-11	27.82[3]	27.72[3]	0.09	0.09				
Averages		21.68	21.64	0.16	0.14	202.8	202.2	0.75	0.72

[1] Some flasks went dry on extractor.
[2] Excessive foreign material in fat.
[3] 3 det. only.
[4] Contains cereal or skim milk.
[5] Jellied fat extract.

about 6 hours in a mechanical convection oven at 101–102°C. They were not extracted with water before being extracted overnight on the Soxhlet apparatus. The petroleum ether complied with **10.69** (*Methods of Analysis*, 7th Ed.). The ether was freshly opened ACS anhydrous in 1 lb. cans and was not re-used. Saponification numbers and unsaponifiable residues were determined on most samples using the pooled fats from the six determinations by each method.

Table 1A gives the results obtained on pork sausage. The average deviations were not significantly different, being 0.26 per cent for petroleum ether and 0.28 per cent for ethyl ether. The averages of unsaponifiable residue values were identical, 0.41 per cent. Saponification numbers were also not significantly different, averaging 209.1 for ethyl ether and 208.2 for petroleum ether. One case of excessive residue in the fat occurred on a sample (9) extracted with ethyl ether. In 5 cases in which the samples were extracted with ethyl ether, one or more flasks went dry during the

night in hot weather. These are noted in the table. Small amounts of residue were often noted in ether-extracted samples on warm, very humid days when the cooling water was cold.

Results from comparison of the two fat solvents on other products are presented in Table 1B. On nine samples containing cereal or skim milk the deviation (0.16 per cent) in the ethyl ether group was twice that of the petroleum ether group (0.08 per cent). These samples are noted in the table. With other products, there were no significant differences between methods. One sample each of frankfurters, bologna, and corned beef hash showed greatly excessive residue in the fat by the ether extraction procedure. Significant amounts of foreign material were frequently found in the ether extracts, but almost never in the petroleum ether extracts.

TABLE 2.—*Comparison of Soxhlet and Acid Hydrolysis fat extraction methods*

PRODUCT	FAT, PER CENT			AVERAGE DEVIATION		
	ETHER	PETR ETHER	ACID HYDROLYSIS	ETHER	PETR ETHER	ACID HYDROLYSIS
Ground Beef 348	12.88	12.85	13.39	0.10	0.13	0.08
A-3	22.54	22.94	23.01	0.35	0.34	0.33
180	18.88	18.99	19.82	0.31	0.19	0.47
16	20.30	20.29	20.47[2]	0.14	0.21	0.23
458	19.52	19.49	19.86	0.14	0.07	0.48
S-2	14.30	14.11	14.55	0.10	0.08	0.08
S-3	14.78	14.69	15.19[1]	0.06	0.09	0.13
S-4	15.27	15.28	15.55[1]	0.15	0.17	0.17
Averages	17.31	17.33	13.73	0.17	0.16	0.25

[1] Twelve determinations.
[2] Ten determinations.

At one time Army specifications listed an acid hydrolysis procedure as the approved method for fat determination in ground beef. Briefly, the method was: Digest ca 2 g prepared sample in 5 ml concd. HCl. Transfer with alcohol to Monjonnier flask. Extract by shaking with 25 ml ethyl ether, followed by 25 ml petroleum ether. Let stand until clear and pour off into tared flask or dish. Repeat extraction three more times. Evaporate solvent and dry fat 1 hr. at ca. 100°C.

This method was compared to the official method and to the petroleum ether extraction method given above. Table 2 lists the results of comparisons on 8 samples of ground beef. Figures reported are averages of 6 determinations except where noted. The acid hydrolysis method averaged 0.42 per cent higher than the official method. Results were more erratic as shown by an average deviation of 0.25 per cent as compared to 0.17

per cent for the official procedure. Much of the increase in fat content found by this method could be due to a larger unsaponifiable residue. For three samples the unsaponifiable residue averaged 0.96 per cent as compared to 0.72 per cent for the Soxhlet ether-extracted fat. In all cases the acid hydrolysis fats were badly darkened; fat oxidation may account in part for the higher values found.

CONCLUSIONS AND RECOMMENDATIONS

In view of the general acceptance of petroleum ether, boiling range 30–60°C., as a solvent for the determination of fat in meat products by the Soxhlet method, and on the basis of the findings reported above, the following changes are recommended as first action:

To **23.6** add: "Petroleum ether (**10.69**) may be used in place of anhydrous ethyl ether, in which case aqueous extraction of carbohydrates before fat extraction is unnecessary. State solvent used in reporting results."

Collaborative studies are recommended on the following:

(1) Use of petroleum ether as a substitute for anhydrous ethyl ether, with possible recommendation of action as an official method.

(2) Study of drying methods for fat determination samples. The present recommended procedure shows evidence of being too harsh by causing excessive fat oxidation.

(3) Analysis of the material determined as fat by the anhydrous ether and petroleum ether methods as to differences in materials dissolved, possible changes in the fat due to peroxide formation in anhydrous ether.

(4) No further study of the acid hydrolysis method seems warranted. The method is unsatisfactory by comparison with the official method.

ACKNOWLEDGMENT

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REPORT ON STARCH IN MEAT PRODUCTS

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The method proposed by Stevens and Chapman (1) for the determination of starch in meat products has been given further study.

In an attempt to shorten the extraction procedure, mild suction was applied. Under these circumstances, the Whatman No. 54 filter paper previously recommended lacked the necessary strength, but a No. 3 Whatman was found to be satisfactory. It was also found that reasonable amounts of skim milk powder could be removed from a meat sample by

shaking only several times during the 10 and 15 minute extraction periods instead of the more frequent agitation previously recommended, although it was not possible to reduce the over-all time of the extraction. There was also a slight increase to 0.25 ml in the meat blank with this modified procedure. The results obtained on amounts of skim milk powder, dextrose, sucrose, and dextrin up to 20 per cent, employing the modified extraction procedure, are shown in Table 1.

These data indicate that skim milk powder, dextrose, and sucrose can be extracted satisfactorily at levels up to 12 per cent and dextrin up to 7 per cent. Since the amounts employed in commercial meat samples would not likely exceed these levels, this extraction procedure appeared

TABLE 1.—*Apparent starch recovery from meat samples containing skim milk powder, dextrose, sucrose, and dextrin*

PER CENT ADDED	APPARENT STARCH FOUND, PER CENT			
	SKIM MILK POWDER	DEXTROSE	SUCROSE	DEXTRIN[1]
1	0.02	0.05	0.03	0.05
3	0.03	0.02	0.03	0.03
5	0.00	0.03	0.05	0.05
7	0.05	0.05	0.03	0.05
10	0.03	0.03	0.00	0.90
12	0.14	0.13	0.00	3.40
14	0.33	0.81	0.03	3.35
20	1.44	1.42	0.17	—

[1] Reagent dextrin (Merck).

satisfactory for these materials. When this method was applied to meat samples containing soy flour and liver, however, the results were less satisfactory. Apparent starch values of 0.24 and 0.65 per cent were obtained on meat samples containing 5 and 7 per cent of soy flour respectively compared to zero values obtained with more frequent shaking. Therefore with samples containing liver or soy flour, it may be necessary to employ frequent or continuous agitation during the 10 and 15 minute extraction periods.

To determine the linearity of the relationship between concentration of dextrose and thiosulfate consumed, an experiment was conducted employing concentrations of dextrose up to 8 per cent. The results are given in Table 2. These data indicate that at levels above 5 per cent dextrose the determination of reducing sugars should be repeated, employing a smaller aliquot of the filtrate and making up to 20 ml with water.

The aliquot of the reduced copper solution taken for the determination of reducing sugars was increased from 40 to 50 ml to facilitate pipetting. Trichloroacetic acid and lead acetate were tested as protein precipitants

TABLE 2.—*Ml of 0.025 N thiosulfate consumed with increasing amounts of dextrose*

PER CENT DEXTROSE IN SAMPLE	ML OF THIOSULFATE CONSUMED
1	3.00
2	5.95
3	9.10
4	12.00
5	14.80
6	17.40
7	19.75
8	22.35

but both were found less satisfactory than phosphotungstic acid and therefore the latter compound has been retained. The concentration of the zinc acetate and potassium ferrocyanide reagents have been decreased to facilitate preparation of these solutions.

The conditions of the hydrolysis were reinvestigated by employing a hot plate in place of the water bath and also by using sulfuric acid instead of hydrochloric acid. Neither of these modifications resulted in any improvement. Experiments conducted since the collaborative results were received, however, have indicated that with potato starch in particular, some non-sugar reducing substances are produced during the prolonged hydrolysis. Therefore, it may be necessary in future work to reduce the period of hydrolysis to 1.25 or 1.50 hours.

The modified method was applied to meat samples containing materials which might be employed as meat binders. A standard was obtained for each product by omitting the extraction procedure and determining reducing sugars after hydrolysis. The materials were added at levels of 1, 3, 5 and 7 per cent and the results are shown in Table 3. These data indicate an average recovery of 102.7 per cent. It appeared from these data that there was some factor or factors which resulted in slightly high recoveries. Nevertheless, these results were sufficiently satisfactory

TABLE 3.—*Recovery of starch from carbohydrate materials added to meat*

WHEAT FLOUR		POTATO STARCH		SOLUBLE WHEAT STARCH		MODIFIED WHEAT STARCH		OAT GUM CONCENTRATE	
ADDED	FOUND	ADDED	FOUND	ADDED	FOUND	ADDED	FOUND	ADDED	FOUND
per cent		*per cent*		*per cent*		*per cent*		*per cent*	
0.72	0.67	0.83	0.88	0.87	0.88	0.83	0.87	—	—
2.16	2.15	2.79	2.61	2.61	2.76	2.79	2.65	—	—
3.60	3.75	4.15	4.53	4.35	4.63	4.15	4.47	2.62	2.66
5.04	5.09	5.81	6.08	6.09	6.20	5.81	5.92	—	—

that it was decided to submit the modified method to collaborative study. The following method was sent to the collaborators.

METHOD

REAGENTS

(a) *Zinc acetate soln.*—Dissolve 12 g of $Zn(C_2H_3O_2)_2 \cdot 2H_2O$ in H_2O and dilute to 100 ml.

(b) *Potassium ferrocyanide soln.*—Dissolve 6 g of $K_4Fe(CN)_6 \cdot 3H_2O$ in H_2O and dilute to 100 ml.

(c) *Copper sulfate soln.*—Dissolve 40.0 g of $CuSO_4 \cdot 5H_2O$ in H_2O and dilute to 1 liter.

(d) *Alkaline tartrate soln.*—Dissolve 200 g of Rochelle salt and 150 g of NaOH in hot H_2O, filter and dilute to 1 liter.

(e) *Standard dextrose soln.*—Dissolve 0.40 g of pure dextrose in H_2O and dilute to 200 ml.

(f) *Starch indicator soln.*—Mix 1 g of powdered starch with 20 ml of cold H_2O. Pour mixt. into 500 ml of boiling H_2O and boil for 10 minutes. Cool and add a few drops of chloroform.

(e) *Phosphotungstic acid soln.*—Dissolve 20 g of phosphotungstic acid in H_2O and dilute to 100 ml. Filter.

DETERMINATION: EXTRACTION AND HYDROLYSIS

Weigh 10 g of finely comminuted and thoroly mixed sample into a 250 ml heat-resistant centrifuge bottle. Add 100 ml of H_2O, 5 ml of freshly prepared zinc acetate soln, and 5 ml of freshly prepared potassium ferrocyanide soln. Stopper tightly and allow to stand for 15 min., shaking vigorously several times during this period. Centrifuge for 15 min. at 1500 r.p.m. Decant the supernatant liquid into a No. 3 Whatman filter paper in a conical funnel employing light suction. To the residue in the centrifuge bottle add 25 ml of a freshly prepared soln containing 1 ml of the zinc acetate and 1 ml of the potassium ferrocyanide solns per 200 ml of soln. Allow to stand for 10 min., shaking several times during this period. At the end of that period centrifuge for 10 min. at 1500 r.p.m. and decant through same filter paper. Repeat this last extn with a further 25 ml of the zinc acetate-potassium ferrocyanide washing soln. Rinse stopper with H_2O.

After centrifuging and filtering, transfer filter paper containing residue to centrifuge bottle and add 90 ml of 1.50 *N* HCl. Suspend bottle in an open boiling H_2O bath, so that the level of the H_2O in the bath is at ca the level of the soln within the centrifuge bottle. Do not reflux. Hydrolyze for exactly 1.75 hrs, maintaining the H_2O level of the bath at its original position, stirring the contents of the bottle occasionally.

Cool immediately. If the detn cannot be finished on the same day, the sample may be allowed to stand overnight at this point. Make just alk. to litmus with 20% NaOH soln (about 27 ml) and then add 10 ml of 1+2 HCl. Transfer soln to a 200 ml phosphoric acid flask or a 200 ml Erlenmeyer flask marked at a vol. of 200 ml. Rinse centrifuge bottle with 15 ml of the phosphotungstic acid soln, followed by several 10 ml portions of H_2O. Make to vol. with the fat layer, if any, just above the 200 ml mark. Stopper, shake, and allow to stand for ca 30 min. Filter soln through a Whatman No. 1 filter paper.

DETERMINATION OF REDUCING SUGARS

Pipet 20 ml of filtrate into a heat-resistant 200 ml Erlenmeyer flask. Add exactly 20 ml of the $CuSO_4$ soln and 20 ml of the alk. Rochelle salt solution. Bring to a boil

within 2 min. with occasional swirling of contents, and continue boiling for one min. Cool immediately under running H_2O, transfer to a 200 ml volumetric flask, make to vol. with H_2O, stopper, and shake.

Pipet 50 ml of above soln into a 200 ml Erlenmeyer flask. Add 25 ml of 10% KI soln and 5 ml of 1+3 H_2SO_4 soln. Titrate with ca 0.025 N sodium thiosulfate soln adding 2 ml of starch indicator and ca 0.5 g of solid NH_4SCN when the yellow I color has almost disappeared. One drop of sodium thiosulfate should change the color from blue to white or faint lilac shade. Carry out a blank detn using 20 ml of H_2O instead of the filtrate, starting at the "Determination of Reducing Sugars." Similarly conduct a detn on 20 ml of the standard dextrose soln starting at the same point.

Calculate % of starch by the following formula:

$$\frac{(A - 0.25) - B}{(A - 0.25) - (C - 0.25)} \times 4 \times 0.9 = \% \text{ starch}$$

where

A = Blank titration in ml.
B = Sample titration in ml.
C = Standard dextrose titration in ml.
0.25 = the average blank obtained on meat samples carried through the entire procedure.
0.9 = factor to convert dextrose to starch.

RESULTS

Samples of potato starch, wheat flour, and skim milk powder were sent to collaborators with instructions to add these materials to ground pork at levels of 2.5 and 5 per cent. The sample of wheat flour and potato starch were found on analysis and calculation of carbohydrate by difference to contain 73.6 and 85.6 per cent of starch respectively. The skim milk powder contained no starch but consisted of approximately 50 per cent of lactose. The results of the collaborative study are shown in Table 4.

These data show that on the average, recoveries were slightly over 100 per cent with all materials. However more than half the analysts reported values in close agreement with the amounts actually present. It may be significant that in 5 out of 6 cases, where appreciable values were reported for the skim milk powder, the results for wheat starch and potato starch were also excessively high.

COMMENTS BY COLLABORATORS

No. 2.—The results appear logical in that the values for the two levels of filler agree fairly well. It would appear that it would be just as accurate and satisfactory to use a 5 ml aliquot for reduction and titrate the entire reduced copper solution. This would save considerable time and would avoid possible errors of transfer and dilution. Suggest the use of $ZnSO_4$-$Ba(OH)_2$ mixture as a protein precipitant.

No. 3.—To avoid transferring from centrifuge bottle to Erlenmeyer or phosphoric acid flask before filtration, would it be feasible to have the centrifuge bottles marked at 200 ml?

No. 5.—Samples A and B foamed excessively and required careful boiling during copper reduction.

TABLE 4.—*Result of collaborative study*

MATERIAL	STARCH ADDED	STARCH FOUND PER CENT — ANALYST 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	AVERAGE STARCH FOUND	RECOVERY
	per cent																	*per cent*	*per cent*
Wheat Flour	3.68	3.63	3.69	3.71	3.84	3.99	3.53	3.60	3.45	3.61	3.96	3.83	4.03	4.29	3.94	4.02	4.06	3.81	103.5
		3.63	3.84	3.73	3.82	3.84	3.49	3.52	3.65	3.65	3.94	3.81	4.01	4.34	3.91	3.93	4.07		
			3.52																
	1.83	1.81	1.76	1.98	1.95	2.07	1.66	1.86	1.70	1.81	2.15	1.38	2.40	2.35	2.25	2.01	2.27	1.94	106.0
		1.80	1.72	1.97	1.89	1.93	1.40	1.87	1.70	1.79	2.16	1.37	2.37	2.52	2.11	1.89	2.26		
			1.86																
Potato Starch	4.28	4.35	4.32	4.45	4.60	4.47	4.16	4.36	4.32	4.38	4.63	3.36	4.74	5.26	4.76	4.90	4.84	4.48	104.7
		4.34	4.32	4.46	4.57	4.58	4.21	4.30	4.29	4.21	4.62	3.20	4.80	5.14	4.73	4.84	4.88		
			4.38																
	2.14	2.21	2.19	2.34	2.35	2.34	2.01	2.27	2.15	2.36	2.53	1.61	2.66	2.63	2.70	2.58	2.63	2.36	110.3
		2.16	2.34	2.34	2.29	2.52	1.92	2.20	2.16	2.22	2.54	2.30	2.70	2.73	2.48	2.50	2.62		
			2.41																
Skim Milk Powder	0	0.09	0.07	0	0	0	0.09	0.02	0.09	0.10	0	0.38	0.49	0.47	0.36	0.51	0.18	0.15	—
		0.07	−0.07	0	0	0	0.07	−0.10	0.07	0.08	0	0.29	0.46	0.43	0.27	0.64	0.08		
			−0.18																
	0	0.06	−0.22	0	0	0	0.09	0	0.02	0.07	0	0.09	0.40	0.06	0.27	0.34	0.19	0.09	—
		0.07	−0.22	0	0	0	0.11	0	0	0.06	0	0.26	0.49	0.03	0.30	0.25	0.17		

No. 6.—Considerable foaming was encountered in samples A and B.

No. 7, 8, 9.—Results are not good as there is a variation between analysts of 10 per cent at the 2.5 per cent level and 5 per cent when 5 per cent starch was added. There are two steps that may cause this variation, that is the hydrolysis with 1.5 *N* acid and the determination of the cupric copper iodometrically. We are satisfied that zinc acetate and potassium ferrocyanide completely precipitates raw starch and removes sugars, but do not believe that cooked starch will be precipitated quantitatively. The method is tedious and requires a great deal of time and apparatus.

No. 11.—Trouble was encountered in the final reducing sugar detn.

No. 12.—We encountered considerable difficulty in the filtration; the first filtration seemed to clog the filter paper and final filtration would barely pass through filter paper. The method as a whole, outside the initial washing and filtering, proceeded in very good analytical order and no difficulties were encountered. We also found for fast calculating purposes on routine work that the following simplification of your formula better explains the operations performed:

$$\frac{A - (B + 0.25) \times 4 \times 0.9}{A - C} = \% \text{ starch.}$$

No. 13.—Samples were shaken continuously on a mechanical shaker. S & S No. 597 filter paper was used, filtering done by gravity. Sodium thiosulfate used was 0.05 *N*. Samples in centrifuge bottles could be chilled enough just before centrifuging so that the fat would be solidified and produce a better separation which would increase ease of filtration. The hydrolysis of the starch possibly could be done in autoclave under pressure and save considerable time. While method is theoretically quantitative, it is difficult and required too much manipulation and time in order to be a good routine determination.

No. 14.—Zinc acetate solution hydrolyzes so rapidly it is difficult to prepare a clear solution of the concentration desired. I recommend the addition of a few drops of acetic acid or filtration of the fresh solution.

No. 15.—Suggest reducing centrifuging time to 5 minutes instead of 15 and 10 minutes.

DISCUSSION

There was little adverse comment regarding the method although two laboratories considered the procedure tedious and time-consuming. The suggestion that the aliquot taken for the reduction should be reduced to 5 ml and the entire copper solution used for the titration has definite merit. Several collaborators have proposed a simplification of the formula used for the calculation and it would appear that the formula proposed by collaborator No. 12 should be adopted. One analyst reported difficulty with the filtration and several others encountered excessive foaming during the determination of the reducing sugars. The cause of these isolated cases of difficulty is not clear.

The point raised by one laboratory regarding the quantitative precipitation of cooked starch requires further investigation. Results on available commercial meat-binders have indicated that these materials are quantitatively precipitated by the foregoing procedure. However, definite information should be obtained regarding the effect on the starch of the

processes involved in the preparation of cooked or partially cooked meat products. All comments and suggestions of collaborators will be considered and investigated if necessary.

RECOMMENDATIONS

It is recommended that this method be given further study. Particular attention should be paid to factors responsible for the recoveries of over 100 per cent reported by a number of collaborators and the behavior of starch which has been exposed to cooking processes.

The assistance of the following collaborators is gratefully acknowledged.

H. R. Cook, U.S.D.A., Bureau of Animal Industry, Meat Inspection Lab., Omaha, Neb.

D. M. Doty and Thomas Keyahian, American Meat Institute Foundation, University of Chicago, Chicago, Ill.

D. Edmond, U.S.D.A., Bureau of Animal Industry, Meat Inspection Lab., New York, N. Y.

J. W. Greer, U.S.D.A., Bureau of Animal Industry, Meat Inspection Lab., San Francisco, Cal.

J. T. Janson, Division of Chemistry, Science Service, Dept. of Agriculture, Ottawa, Ontario, Canada.

J. B. Jones and Miss E. J. Beverly, Food and Drug Laboratories, Dept. of National Health and Welfare, Winnipeg, Man., Canada.

G. E. Mack and E. W. Corck, Food and Drug Laboratories, Dept. of National Health and Welfare, Toronto, Ont., Canada.

R. A. Maxwell, U.S.D.A., Bureau of Animal Industry, Kansas City 10, Mo.

F. D. Roach, U.S.D.A., Bureau of Animal Industry, Meat Inspection Lab., St. Louis, Mo.

H. O. Tomlinson, F. R. E. Davies, N. Bluman, Food and Drug Laboratories, Dept. of National Health and Welfare, Vancouver, B. C.

E. F. Whyte and L. B. MacIsaac, Food and Drug Laboratories, Dept. of National Health and Welfare, Halifax, N. S.

Ernest S. Windham, Army Medical Service Graduate School, Army Medical Center, Washington 12, D. C.

REFERENCE

(1) Stevens, F. J., and Chapman, R. A., *This Journal*, **35**, 345 (1952).

No report was made on: creatin in meat products; horsemeat in ground meat (chemical); or on horsemeat in ground meat (serological).

The contributed paper entitled "The Use of Serological Methods in the Regulatory Control of Foods" was published in *This Journal*, **36**, 107 (February, 1953).

REPORT ON EXTRANEOUS MATERIALS IN FOODS AND DRUGS

By KENTON L. HARRIS (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Referee*

RECOMMENDATION*

The Report on sediment tests in milk and cream includes several recommended changes involving the description of a sediment filtering apparatus and the necessary accompanying changes. There is a minor change in the description of the type of sediment pad and the performance limits of the pad. The report presents the results of a rather extended investigation of fine sediment and recommends that the revised method, to be designated as **35.9(c).**, be adopted, first action. The Referee concurs in these recommendations. In the Associate Referee's report there is some indication that the description of the filtering apparatus may warrant further attention. The Associate Referee may wish to consider this point.

The report on extraneous materials in vegetable products mentions two pieces of work which will be continued as material becomes available. The Referee recommends that this work be continued.

The report on extraneous materials in nut products reports a new procedure for peanut butter to replace **35.26** and the comparable changes in **35.25.** Collaborative results are presented and the Referee concurs in the first action recommendation of the Associate Referee.

The report on extraneous materials in drugs, spices, and miscellaneous products recommends that the methods reported in *This Journal*, **35,** 328–330 (1952) which were suggested to replace **35.83, 35.84, 35.85, 35.86,** and **35.87** be adopted, first action. The Referee concurs in this recommendation. A method for ground capsicums is reported. This method is designed to replace the present **35.87** procedure. It is recommended that collaborative results be obtained on this procedure during the coming year.

The report on extraneous materials in dairy products reports further work in this field and recommends that the work be continued, and that collaborative samples be sent out when further developments are encountered. The Referee concurs in this recommendation.

The title of the report on extraneous materials in cereal grains, cereal products, and confectionery more adequately covers the field than did the old title, "... baked products, prepared cereals, and alimentary pastes" and it is recommended that this title be used hereafter. The Referee concurs in all of the Associate Referee's recommendations, as follows: that the cracking-flotation procedure and the pancreatin-flour procedure be studied collaboratively; and that the method for preparing

* For report of Subcommittee D and action of the Association, see *This Journal*, **36,** 62 (1953).

gasoline saturated isopropyl alcohol be added as a part of section **35.4.**

It is recommended that an Associate Referee be appointed to work on methods for the identification of insect contaminants of food and drug products and determine if there are ways to make concise descriptions of fragments from these insects.

REPORT ON EXTRANEOUS MATERIALS IN DRUGS, SPICES, AND MISCELLANEOUS PRODUCTS

By WILLIAM V. EISENBERG (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

It is recommended* that methods **35.83, 35.84, 35.85,** and **35.86** reported during the October 1950 meetings and published in *This Journal,* **35,** 328–330 (1951), be adopted as official, first action.

These methods have been used by Food and Drug Administration analysts for the past two years and have given consistent results. Experience obtained in the use of the new methods has shown that the proposed methods permit the use of larger samples, give cleaner separations, and more complete recoveries.

A change in Method **35.87** for extraneous materials in ground capsicums has been suggested as a result of collaborative work by analysts of the New York District laboratory of the Food and Drug Administration. The change in the method involves the addition of one step calling for boiling the pancreatin digested material in the trap flask before trapping off with gasoline. This gives a cleaner separation of extraneous material from the plant tissues.

These methods are published as part of *Changes in Methods, This Journal,* **36,** 89–90 (1953).

REPORT ON EXTRANEOUS MATERIALS IN DAIRY PRODUCTS

By DOROTHY B. SCOTT (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

The staining technique for plant and dung fragments in dairy products has been studied and improved during the year. This report presents the results of these studies. It also reports the results on four collaborative samples and continues the plan of giving the histological botanical background necessary to a basic understanding of the problem.

In previous reports† of the Associate Referee on the study of plant

* For report of Subcommittee D and action of the Association, see *This Journal,* **36,** 62 (1953).
† *This Journal,* **34,** 350 (1951); **35,** 330 (1952).

and dung fragments in dairy products, the chemical composition of the plant fragments before and after digestion in ruminant animals was discussed. A method of staining plant and dung fragments to aid in observing the characteristics of fragments before and after digestion was given.

HISTOLOGICAL BACKGROUND

The plant cell consists of a cell wall and living protoplasm, a viscous, colloidal fluid 75–90 per cent water, and solids.

The cell wall is pronounced when viewed microscopically and remains relatively rigid. In cells with thin walls, there are three layers, each of two adjacent cells producing a primary wall cemented together with an intercellular layer of complex pectic substances commonly called pectin (middle lamella). These thin walls are in the immature tissues and in the soft parts of plants, such as fleshy stems and leaves.

Hard plant materials owe their characteristic properties to the relative thickness and density of a secondary wall which adds two or more layers in addition to the three layers as the cell matures. Lignin infiltrating the wall layers causes the cell walls to become stiff and less readily digested.

The cytoplasm appears granular in non-living tissue because of the precipitation of the cell contents. The plastids which it contains are of three kinds. The chloroplasts contain chlorophyll. The chromoplasts contain specialized pigments, the carotenoids. The third type of plastids are leucoplasts which are colorless. They serve as centers of starch formation. The nucleus is also in the cytoplasm.

The remaining portion of the cell consists of the vacuoles which are spaces within the protoplasm filled with fluid containing many substances in solution.

Plant cells become specialized and the aggregations of these cells form the tissues which in turn form the plant.

Xylem and phloem comprise the vascular tissue of the plant. Xylem contains vessels composed of cells placed end to end and connected by perforations, tracheids which are elongated, thick-walled cells with tapering ends and pitted walls, and fibers which are greatly elongated supporting cells, tapering at both ends. The phloem is composed of thinner-walled sieve tubes, fibers and parenchyma cells. The cross cells of the bran of cereals are arranged in parallel, have thick-beaded walls, and are transversely elongated. The cross cells are covered with a waxy layer and contain no chlorophyll. The glumes, lemmas, and palets from cereals have elongated, wavy-walled cells in the epidermis. The cells have thick-silicified and lignified walls. All of these heavy-walled tissues are found in dung.

In the digestive system of the ruminants, the bacteria, enzymes, and juices act on the soft parts of plants and immature tissues which are assimilated for nourishment. The hard, usually lignified and mineralized,

portions which are resistant to the digestive processes are excreted in the dung.

Other tissues whose cells are composed of cellulose thickened and combined with compounds impervious to the digestive processes of the ruminants are found in dung. The compounds are cutin, suberin, resins, and other fatty or waxy substances. For example, cork with suberized cellulose cell walls is resistant and impermeable. It is the secondary tissue formed from the cork cambium layer and is found in a few herbaceous plants.

When large fragments, not thoroughly masticated, pass through the digestive system of the ruminants, the bacteria, enzymes, and digestive juices do not always act on the inner portions of the fragment. The surface tissue consisting usually of parenchyma cells may be digested. Frequently, plant hairs attached to bran tissues are in dung, and single hairs occur in large quantities. Because of their thick walls, they are not digested.

The vascular elements of stems are the most abundant constituents of dung, together with smaller amounts of vascular bundles or veins from leaves. Free spirals consisting of secondary walls of vessels released after the digestion of the primary walls may be numerous, and jagged ends may be caused by the digestion of pectic substances in the intercellular layers and the primary walls. This digestion also often makes the tissues of dung fragments easy to tease apart.

A dung fragment may consist of several kinds of tissue. Characteristics of each may be found in a single fragment.

Raw dung from all ruminants contains a large quantity of mucilage which serves as an important diagnostic characteristic of plant fragments from raw materials and manufactured products.

It consists of the substance produced by the breaking down of the cell walls and cell contents and a large number of dead bacteria, worn out cells from the intestinal lining, dirt, mucus, sometimes pollen grains, mold filaments, and spores. It often binds together the digested plant fragments. When the mucilage or slime on the surface of the fragments is studied at 100–400×, the various components may be seen. When dung fragments are separated from dairy products, it is a rare thing to find no surface contamination of mucilage, slime, or dirt in varying quantities.

The mucilage is present in large quantities on dung fragments from separator sludge, filters from dairy plants, and sediment pads used in testing milk and cream. Dung fragments from cheese will have less mucilage.

It is important to study both the inside and outside of the fragment. The surface deposit has important characteristics as well as the cell walls, cell contents, and internal structure of the plant tissues.

STAINING TECHNIQUE AND INTERPRETATION

To make the characteristics both inside and outside of fragments more easily observed, the Graichen-Harrow iodine staining technique has been developed. The staining technique is empirical and is still undergoing study.

1. Immerse fragments in a few drops of 50% chloral hydrate soln in a small beaker or hanging drop glass slide.
2. Bring to incipient boiling on a hot plate or micro burner.
3. Cool and transfer with water to a fritted glass funnel.
4. Rinse thoroly with water. Turn off suction. Add a few drops of Lugol's iodine soln, immersing all fragments.
5. Allow to stain for five min.
6. Drain and rinse thoroly with water.
7. Turn off suction. Immerse in a 1+9 diln of the Graichen-Harrow stain: Eosin, 0.2%; Wool violet 4BN (C. I. 698); 0.05% Niagara Sky Blue 6B (C. I. 518); 0.15% in water.
8. Stain for 15 minutes.
9. Drain and wash thoroly with water.
10. Wash with 50% alcohol to destain.
11. Wash with 95% alcohol.
12. Wash with 10% acetic acid soln.
13. Wash with water.
14. Add 3% hydrogen peroxide and let stand immersed for five minutes or longer.

The fragments may be transferred to a filter paper and examined using water for wetting the paper.

If desired, they may be allowed to dry at room temperature and then mounted in a permanent medium. The oxidizing treatment with hydrogen peroxide makes the fragments retain their stain in water and in permanent mounting.

The iodine will give the lignified cellulose a light brown color. Cork tissue, glumes, lemmas, and palets of the cereals will also frequently be stained brown with the iodine. Cell walls, adhering tissue and some cell contents may take the Graichen-Harrow dyes. When starch is formed in the leucoplasts, the iodine will give the familiar dark blue color.

The red, purple, and blue dyes in the Graichen-Harrow stain are not entirely selective. In general, the edible nutrients in the undigested plant fragments will stain blue and purple. Chlorophyll, the carotenoid pigments in the cytoplasm, and the anthocyanins in the vacuoles may retain their characteristic natural colors or may stain deeply, usually purple.

When browning takes place in the plant tissue, due to the enzymic action of bacteria and molds in decayed and weathered plant fragments or in silage, the staining may be masked at low power. However, when viewed at 100–400×, some staining of cell walls and cell contents may be seen.

The important characteristic of all plant tissue not digested in the ruminant's system is the quantity of cell contents. Decay of plant tissues

does not have the same drastic effect on the cell contents, and usually the cell walls, as does the digestive processes of the ruminants.

The mucilage at 30× appears as a light brown, granular, amorphous substance. It may be slightly pink or not stained at all. Fine mold such as oospora found in dairy products often stains a light blue. Other fine mold filaments may stain red or purple. The deep brown large mold filaments and spores associated with rot and decay in plant tissues do not usually take the stains.

Cheese fragments or curd which may adhere to the fragments will be deeply stained red or orange.

The vascular tissue often retains a small amount of stain and may be stained lightly red or purple.

The interpretation of the results of this staining technique should not be due entirely to the depth of color seen when the fragments are viewed macroscopically or at low power microscopically, but should include a study of the components of the plant tissue that take the stain. In general, primary walls consisting of pectic substances and cellulose absorb some or all of the dyes of the Graichen-Harrow stain and the lignified cellulose of the secondary walls absorbs the light brown color of the iodine. When there is a large quantity of cell contents, the tissue will be stained much deeper. Vascular tissue, which normally does not contain cell contents

TABLE 1.—*Comparison of plant fragments from dung and undigested fragments*

DUNG FRAGMENTS	UNDIGESTED PLANT FRAGMENTS
Lignified cellulose—brown iodine color	Lignified cellulose—brown iodine color
Cells walls—not stained or stained very slightly pink or purple	Cell walls—deeply stained and distinctly outlined—blue, red or purple
Little or no cell contents to take the stain	Cell contents—deeply stained
No starch	Starch—stains dark blue
Parenchyma cells adhering to conductive elements devoid of cell contents	Parenchyma cells contain stained cell contents
Ends of fragments jagged and separated	Ends of fragments remain attached
Few, if any, pigments	Pigments normal color or deeply stained, usually purple
Glumes, lemmas, palets of cereal grains —brown iodine color or not stained	Glumes, lemmas, palets, lignified portions stained light brown, some tissues attached stained red or purple
Cross cells translucent—not stained	Cross cells not stained

when mature, will not take much of the Graichen-Harrow stain but the lignified cell walls will take the light brown iodine color and only the adhering tissue will stain red, blue, or purple.

On the surface, the components of the mucilage, slime, or dirt is an important factor in determining the origin of the fragment.

In summation, a knowledge of the histology of the plant material and a detailed examination of the surface particles are necessary in determining whether or not the fragment is from dung or compost, or is undigested plant tissue (see Table 1.)

COLLABORATIVE RESULTS

Four samples of extraneous material in butter oil were distributed to ten collaborators. Their findings are as follows (Table 2):

TABLE 2.—*Results of analysis of four samples by ten collaborators*

COLLABORATOR	SAMPLE 1	SAMPLE 2	SAMPLE 3	SAMPLE 4
MATERIAL ADDED	DUNG	DUNG & PLANT	PLANT	PLANT & COMPOST
A	Dung	Dung & plant	Plant	Plant compost
B	Dung at 30×, decayed plant at 75×	Dung & plant	Plant	Plant
C	Dung, few plant	Plant	Plant	Plant
D	Dung	Dung	Mostly plant, few dung	Mostly plant, few dung
E	Dung	Dung & plant	Dung & plant	Dung & plant
F	Dung	Dung & plant	Plant	Plant & dung
G	Dung	Dung	Plant	Plant, few dung
H	Dung, few plant	Dung, few plant	Dung, plant	Dung, plant
I*	Some dung	Dung & plant	Plant	Plant
J	Plant	Dung & plant	Plant, few dung	Plant, dung

* See comments from this collaborator.

COMMENTS OF COLLABORATORS

Collaborator B.—Sample 1 when examined at 30× resembled manure fragments. However, many of the fragments do not appear covered with mucilage and at 75× much cell structure seemed evident. Upon staining, many fragments took on a light purple tint indicating staining of cellular material. Therefore, I would conclude that the majority of the fragments in this sample are actually decayed plant fragments and very little, if any, manure.

Sample 2 consisted mainly of manure fragments. Staining showed further evidence of this being true.

Collaborator C.—By the staining procedure, indications are that the 6 plant fragments in sample 1 are all dung, but proof is not conclusive so they are counted as plant fragments. It appears that samples 3 and 4 contained ground feeds and sample 2 contained partially decomposed (oxidized) plant material. The fragments in samples 3 and 4 dyed well to blue and purple color, while those in sample 2 stained to a brown color. Fragments in sample 1 stained very little, if any.

Collaborator F.—The appearance of the plant fragments in sample 2 was different from those in the other samples. Before staining, they were a much darker brown color in general. Some of the clumps of fragments held together by a mucilagenous type of material and some of the single fragments showed specks of black foreign material. At high magnifications, some fragments showed mold filaments.

Collaborator I did not have time to stain the fragments and the analysis is based upon morphological characteristics visible without staining.

Sample 1: Some manure fragments present. Other fragments translucent but no mucilage present. Some fragments had mucilage but looked more like compost and did not disintegrate when probed with needle.

Sample 4: Plant, except for three small fragments that were suspicious. These had mucilage and broke up when probed but were too small for other identification necessary to class them as manure.

COLLABORATORS

I wish to thank John Bornmann, Chicago District; Juanita Breit, Minneapolis District; Luther G. Ensminger, Cincinnati District; Helen T. Hyde, San Francisco District; Robert E. O'Neill, Atlanta District; J. E. Roe, Denver District; George Schwartzmann, New York District; Harold E. Theper, St. Louis District; Shirley M. Walden, Baltimore District; and A. H. Wells, Los Angeles District, all of the U. S. Food and Drug Administration, for their collaboration.

It is recommended*—

1. That the staining technique for dung and plant fragments in dairy products be further studied with a view to obtaining more easily interpreted results.
2. That samples be submitted to collaborators if an improved staining technique is developed.

REPORT ON EXTRANEOUS MATERIALS IN NUT PRODUCTS

By MARYVEE G. YAKOWITZ (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

The present method for the determination of light filth in peanut butter has been the subject of criticism by many analysts from time to time, from the standpoint of difficulty in handling, filth separation, and recovery. At the suggestion of Mr. J. E. Roe of Denver District, Food and Drug Administration, a modified method involving a pancreatin digestion procedure has been developed by the Associate Referee and subjected to collaborative testing. The method appears in *This Journal*, **36**, 88 (1953).

Duplicate 100 g samples of peanut butter, each containing 20 rodent hair fragments (1–2 mm) were examined as unknowns by six collaborators using (1) the present method (**35.25** and **35.26**) and (2) the proposed pancreatin digestion procedure given above. The results obtained are shown in Table 1.

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* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 62 (1953).

TABLE 1.—*Recovery of rodent hairs from peanut butter*

COLLABORATOR	PRESENT METHOD (35.25 AND 35.26)			PROPOSED PANCREATIN DIGESTION PROCEDURE		
	1ST EXTRACT	2ND EXTRACT	TOTAL	1ST EXTRACT	2ND EXTRACT	TOTAL
1	1	9	10	16	2	18
2	13	2	15	20	0	20
3	0	0	0	4	10	14
4	12	1	13	13	4	17
5	6	6	12	5	1	6
6	6	2	8	18	2	20
Average recovery			9.7			15.8
Average per cent recovery			48.5			79.0

Four collaborators expressed a definite preference for the proposed procedure, particularly because of the ease of separation and the cleanliness of the filter papers for microscopical examination. Collaborator 5 stated that the better recovery obtained by him using the present method might perhaps be due to a comparative lack of familiarity with the pancreatin extraction procedure. The results show an average of 30 per cent additional rodent hair recovery by the proposed pancreatin digestion procedure.

RECOMMENDATION

It is recommended*—

(1) that Method **35.25** for "WIIR" and excreta, the changes in which affect only the subsequent determination by **35.26**, remain official, first action.

(2) that Method **35.26** for light filth in peanut butter be replaced by the above modified method, which includes the pancreatin digestion procedure. This method should be adopted, first action.

ACKNOWLEDGMENTS

The Associate Referee wishes to thank the following analysts of the Food and Drug Administration who kindly cooperated:

Manion M. Jackson, Philadelphia District; Flora Y. Mendelsohn, Los Angeles District; J. Frank Nicholson, Washington, D. C.; Aldrich F. Ratay, Cincinnati District; J. E. Roe, Denver District; John P. Traynor, Baltimore District.

* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 62 (1953).

REPORT ON EXTRANEOUS MATTER IN CEREAL GRAINS, CEREAL PRODUCTS, AND CONFECTIONERY

INTERNAL INFESTATION IN WHEAT

By J. FRANK NICHOLSON (Food and Drug Administration, Federal Security Agency, Washington 25, D.C.), *Associate Referee*

A method for the determination of internal insect infestation of wheat and which, with slight modification, may be adaptable to other cereal grains is as follows:

METHOD

Mix grain to be examined by passing through a Jones sampler, recombining the sepns before each pass. After mixing, separate slightly more than 100 g and weigh 100 g. Brush the sample (a small amount at a time) on a 5–8″ No. 12 sieve, using a stiff bristled brush to work the insects through the sieve as completely as possible.

Grind the screened wheat in a Labconco, or equivalent, mill set at 0.061 inch. (Before grinding, dry damp or tempered wheat in a forced draft oven at 70–80°C. for 1 hr., or for ca 2 hrs if no draft is used.) Transfer the cracked wheat, including any residue in the mill, to a 2-liter trap flask. Trap as in **35.4(a)**, as revised *This Journal*, **35**, 94–95 (1952), using, as the solvents, 60% isopropyl alcohol saturated with gasoline, and gasoline, and filter on 10XX bolting cloth. If on trapping off a starchy residue remains, hydrolyze as in **35.4(a)**. Examine as under **35.4(b)** except 15× may be used instead of 20× as the lower limit of magnification. Count only whole insects, insect heads, cast skins, and head capsules.

It is recommended* that this method be studied collaboratively during the coming year.

The use of X-rays for the determination of internal insects in a number of materials such as wheat, corn, popcorn, coffee, beans, spices, etc., is under study and will be reported later.

The pancreatin digestion method for extraction of insect fragments from flour has been the subject of discussion by analysts regularly using it, and it is recommended* that **35.29(a)** be revised as follows:

Weigh 50 g flour into beaker, stir into a smooth slurry using pancreatin soln made as in **35.2(d)**† diluted with 100 ml of H_2O. Dilute slurry with H_2O to make a final volume of ca 400 ml. Adjust to *p*H 8 using Na_3PO_4 soln. Readjust the *p*H after ca 15 min. and again in ca 45 min. Stir in 3 drops of formaldehyde soln U.S.P. and digest for 16–18 hrs. Transfer to a Wildman trap flask and extract as in **35.4(a)** as revised in *This Journal*, **35**, 94–95 (1952). Stir for 1 min. instead of 2 as given in the above revision, using gasoline and water as the solvents. Catch combined trappings and rinsings in a beaker and transfer to a 2 l trap flask. Trap off as above. If considerable starchy material is in the extract, hydrolyze with HCl as in **35.4(a)**. Examine as in **35.4(b)**.

Section **35.4** describes some techniques commonly used in Chapter **35** methods. It is recommended† that the following description be added to this section:

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* For report of Subcommittee D and action of the Association, see *This Journal*, **36**, 62 (1953).
† As revised, *This Journal*, **35**, 94 (1952).

Saturation of 60 per cent Isopropyl Alcohol with Gasoline.—Add ca 150 ml gasoline to each liter of technical isopropyl alcohol. Mix and dilute with water to 60 per cent. Allow to stand until comparatively clear. Siphon the alcohol from beneath the gasoline layer, and filter.

REPORT ON EXTRANEOUS MATERIALS IN VEGETABLE PRODUCTS

By FRANK R. SMITH (Food and Drug Administration, Federal Security Agency, Washington 25, D.C.), *Associate Referee*

There is little to report this year in the section on extraneous materials in vegetable products. No further work was done on the method for fly eggs and maggots in spaghetti sauce because of lack of suitable material. Further work on this method will be carried out when suitable material is available.

C. D. Shiffman of Atlanta District, Food and Drug Administration, has reported a revision of **35.73** (weevils in peas and beans) which shows promise of being an improvement over the present method. Further work will be done on this method as material becomes available.

REPORT ON SEDIMENT TESTS IN MILK AND CREAM

By CURTIS R. JOINER (Food and Drug Administration, Federal Security Agency, St. Louis, Mo.), *Associate Referee*

Last year a method (1) for the preparation of fine standard sediment disks was presented, but insufficient collaborative work had been done to recommend its adoption. This year the method was again studied collaboratively.

COLLABORATIVE WORK

Seven chemists in six different laboratories prepared duplicate sets of nine pads each from the standard sediment mixture supplied by the Associate Referee. Four of them also prepared his own standard mixture and made up pads from that. The work and comments of each collaborator are discussed below.

Collaborator 1.—This analyst reported that he had difficulty in getting the sediment evenly distributed over the pad. He made five or six pads for each concentration and selected the two best ones. The disks he submitted were satisfactory.

Collaborator 2.—The pads submitted by this analyst were unsatisfactory because of uneven distribution of sediment. The appearance of the pads indicated that the filtering apparatus used was unsuitable for the purpose. Correspondence proved this to be the case. He devised a filtering apparatus that was acceptable and prepared pads that were satisfactory

as to distribution of sediment. However, they were made with thick, loose-textured disks, and some of the sediment was buried beneath the surface of the disks. A partial set of pads prepared with thinner, harder-surfaced disks was entirely satisfactory. This analyst recommended that a description of the filtering apparatus be included in the method.

Collaborators 3, 4, and 5.—These analysts made no comments about difficulties, and the pads they submitted were satisfactory.

Collaborator 6.—Two sets of standard disks were submitted by this collaborator, who reported that five disks of each standard had been prepared and the best two of each five selected. Three of the 18 disks in the two sets were classed as unacceptable by the Associate Referee because of uneven distribution of sediment. The analyst used a filtering apparatus with a 45 degree funnel and suggested that this might have caused the sediment to concentrate around the periphery of the pad rather than to settle evenly over the entire area. Later, the analyst reported that this funnel had been replaced with an 80 degree one, and ten satisfactory disks had been prepared.

TABLE 1.—*Sediment passing through disks*

TYPE OF DISK	COLLABORATOR NUMBER				
	1[a]	2[b]	3[b]	4[a]	5[c]
	mg	*mg*	*mg*	*mg*	*mg*
Cream disk A	1.6			2.8	2.8
Milk disk A		1.6		2.6	2.4
Cream disk B			4.9	2.1	2.7
Milk disk B	1.5		1.3	1.5	2.1
Cream disk C				2.4	1.7

[a] Average of four disks.
[b] Average of three disks.
[c] Average of two disks.

Collaborator 7.—Duplicate sets of pads prepared from the Associate Referee's mixture were all acceptable. Of the 18 disks made up from the collaborators' own mixture, four were rated as unacceptable by the Associate Referee because of uneven distribution of sediment over the surface of the disks.

Five of the collaborators measured the amount of sediment passing through the pads as directed in the last paragraph of the method. The results are listed in Table 1. The values given are for 12 milligram pads.

DISCUSSION

Standard disks prepared from different sediment mixtures differed slightly in appearance. When viewed under the low power microscope some differences in particle size and shape were apparent. However, these differences in general appearance were insignificant.

The only real difficulty seemed to be that of getting the sediment fairly

evenly distributed over the disk. This collaborative work has demonstrated that if a satisfactory filtering device is available, only sufficient practice with proper attention to the details of the method are needed to obtain satisfactory results. One hundred per cent acceptable results are not necessary and probably are not attainable. The unsatisfactory pads should be discarded and new ones made.

In spite of the trouble experienced by two of the collaborators and the suggestion made by one of them, it seemed unwise to include in the method a detailed description of any particular piece of apparatus, because there are many different types of devices that can be used successfully. There are in use in this laboratory one custom built filtering apparatus* and one made in the laboratory. Both work satisfactorily, but the former is more convenient to use. The essential requirements and performance standards of a filtering apparatus were written and submitted to the collaborators for comment. The six who replied were in unanimous agreement that the description should be a part of the method. Several thought it should be more detailed. The description is given below under recommendations.

Thorough wetting of the disk before filtering the sediment suspension is essential. Four of the collaborators remarked about this. One used a dilute solution of aerosol and one a 25 per cent alcohol solution to wet the pads. Another satisfactory procedure is to hold the pads under a stream of water from the faucet for a few seconds before placing them in the filtering device.

Sediment disks from three different manufacturers† were used by the collaborators. Based on the limited number of measurements of the amount of sediment that passed through the disks (Table 1), there is not much difference between the three manufacturers' products. The one value of 4.9 milligrams for cream disk B appears to be out of line. It is possible that this represented an unusually porous lot of disks. The standard pads submitted by this collaborator were made with milk disks and not with the cream disks. Collaborator No. 7 submitted values of 6.4 and 8.3 milligrams for cream and milk disks B, respectively. He used the cream disks in preparing his standards, and they were comparable in appearance to those prepared by other collaborators with the same type of pads. He reported that he had dried the filter papers two hours and had not checked them for constant weight. Work by several analysts in this laboratory has indicated that it occasionally takes three hours or more to dry the papers to constant weight. For this reason these high values were not included in the table. At least one collaborator combined the three filtrates and had only one paper to weigh. This procedure should reduce the error of weighing.

* Made on special order by Precision Scientific Co., Chicago.
† Langsenkamp-Wheeler Brass Works, Inc.; Johnson & Johnson, Filter Products Division; and Sediment Testing Supply Co.; all in Chicago.

The amount of sediment passing through the pads, about 20 per cent, is rather large. However, it is reasonable to assume that when the same type of disk is used for a field sediment test that a comparable amount of the fine sediment will be lost. If the sediment on the test disk is predominantly coarse, then the coarser standard disks should be used for grading it.

The relatively thin "creamtest" disks are usually more satisfactory for the fine standard pads than the "milktest" disks because more of the sediment gets embedded in the thicker pad. Sometimes this gives a noticeably lighter appearing disk. Fortunately, the "milktest" disks on the market today are superior to those that were sold several years ago.

The work mentioned in last year's report about the preservation of standard sediment disks by high pressure lamination in clear plastic was continued. The firm* that was doing the work has developed a process that appears to be successful. The disks were first coated with a solution of the plastic to be used and then glued in place on a strip paper with the same plastic. The paper with the standard disks attached was embedded in the plastic.

SUMMARY

Five collaborators submitted acceptable standard disks. One of these had difficulty in devising a suitable filtering apparatus.

Two collaborators submitted standard disks of which 20 to 25 per cent were unacceptable. In one case the trouble was caused by the filtering apparatus being used. When this was corrected, satisfactory standard disks were prepared.

The collaborative work indicated that some description of the filtering apparatus should be included in the method. Since the same type of device is used for preparing both types of standard disks, it is logical to insert the description just ahead of the present par. **35.9(c)**(2). The insertion of the description will necessitate a few minor editorial changes in **35.9(c)**.

The tentative tolerance for sediment passing through the disks included in the method last year should be raised to be in line with the majority of the results obtained by the collaborators.

The fine standard sediment disks, if adopted by this Association, should be used in conjunction with the present standards. For grading a particular test disk, the type of standard that it more nearly resembles should be used.

RECOMMENDATIONS

It is recommended†—

(1) That the following description be adopted, first action, and be

* Tires Inc., St. Louis, Mo.
† For report of Subcommittee D and action of the Association, see *This Journal*, 36, 62 (1953).

designated as **35.9(c)**:

Sediment disk filtering apparatus.—Apparatus must hold 1¼″ sediment disk and have effective filtering area 1⅛″ in diameter. This 1⅛″ area must be unobstructed except for wire screen or perforated plate and wire screen support for filter disk. Apparatus should be supported in neck of filtering flask so vacuum can be used for rapid filtration or flask outlet closed to practically stop filtration. Apparatus should have ca 80° funnel with min. capacity of 80 ml and max. capacity of 450 ml. Test apparatus by filtering H_2O suspension of carbon. Disk should have clean border. When sediment suspension is filtered, sediment should be evenly distributed over disk with no pattern formation.

(2) That the text of the first action method designated as **35.9(c)** be changed as follows, and that the revised method be adopted, first action, and be designated as **35.9(d)**: The heading should be changed to "*Preparation of coarse standard sediment disks.*"

Beginning with the eighth line of the third paragraph, the sentences, "Mix thoroughly and pass mixture through standard sediment disk in filtering device having filtering area measuring 1⅛″ in diameter. Pour milk gently down side of filtering apparatus and filter with very little or no suction," should be changed to: "Mix thoroughly and pass mixture through standard sediment disk in filtering apparatus (c). Pour milk gently down side of funnel and filter with very little or no suction."

(3) That the following changes be made in the method for the preparation of fine standard sediment disks (3) presented last year and the revised method be adopted, first action, and be designated as **35.9(e)**:

In the second line of the second paragraph, insert "(c)" after word "apparatus."

Beginning in the sixth line of the second paragraph the sentence, "With filter flask air outlet closed to prevent filtration, mix diluted aliquot and pour into funnel fitted with a wet standard disk that passes test given below," should be changed to, "With filter flask air outlet closed to prevent filtration, mix diluted aliquot and pour into filtering apparatus (c) fitted with a thin, smooth, hard surfaced, wet, standard disk that passes test given below."

In the next to the last line, "2.2 mg" should be changed to "2.8 mg." Add the following sentence to the end of the paragraph: "Fine standard sediment disks may be prepared and used in any range between 0 and 14.0 mg."

(4) That the present **35.9(d)** be changed to **35.9(f)**.

ACKNOWLEDGMENT

The writer wishes to thank the following members of the staff of the Food and Drug Administration for participating in the collaborative work: Helen T. Hyde, San Francisco; George E. Keppel, Minneapolis;

T. E. Strange, Portland; H. W. Conroy, Kansas City; Frank H. Collins, Cincinnati; Mary A. McEniry and Richard F. Heuermann, St. Louis.

REFERENCES

(1) JOINTER, CURTIS R., *This Journal*, **35**, 340 (1952).
(2) *Ibid.*, p. 99.
(3) *Ibid.*, p. 342.

No report was made on extraneous materials in fruit products, in beverage materials, or in miscellaneous materials.

REPORT ON MICROBIOLOGICAL METHODS

BY GLENN G. SLOCUM (U. S. Food and Drug Administration, Federal Security Agency, Washington, 25, D.C.), *Referee*

The status of the work of the Associate Referees on Microbiological Methods is as follows:

Sugar.—The method is entirely satisfactory for the detection and estimation of thermophilic bacteria in sugar and has been used extensively in various laboratories for that purpose. It must, however, retain its status as "First Action" until further collaborative studies are conducted.

Canned Vegetables.—This method, also first action, is employed widely with satisfactory results. It requires further collaborative study to serve as a basis for a recommendation of its adoption as an official method.

Canned Fruits and Other Acid Canned Foods.—The tentative methods described in the Sixth Edition proved unsatisfactory and were dropped from the Seventh Edition of *Methods of Analysis*. It seems unlikely that general methods applicable to all types of acid canned foods can be devised and it will be necessary to further subdivide this category and develop separate methods for different classes of products because of the variation in the microorganisms primarily responsible for spoilage. The Associate Referee has conducted some studies with tomato products. In view of the commercial importance of canned tomato products and since the microbiology of spoilage of these products is fairly well known, the development of official methods for their analysis should receive first attention.

Canned Fish.—Active spoilage of canned fishery products is a rare occurrence and the need for special methods differing in a material respect from the methods for canned vegetables (non-acids) is not apparent. The production of fresh and frozen seafood products has expanded enormously in recent years and there is much more need for the development of microbiological methods for such products as crab meat, shellfish, fresh and frozen fish fillets, etc., than for canned fishery products. This subject should be expanded to include all fish and fishery products.

Canned Meats.—There has been no activity on the development of microbiological methods for these products. The need for methods for canned meats as well as other meat products will be explored and a recommendation for future course of action made later.

Nuts and Nut Products.—The Associate Referee has conducted further studies since the tentative method in the Sixth Edition was dropped. A definitive method has not yet been developed.

The American Public Health Association has long been interested in the development of a Manual for the Microbiological Examination of Foods. Dr. Goresline, Chairman of this A.P.H.A. committee under the Coordinating Committee on Laboratory Methods, has collected material for the first edition of such a manual which probably will be published by that Association as "Recommended Procedures," rather than as "Standard Methods." Coordination of A.O.A.C. and A.P.H.A. methods is, of course, essential to avoid outright conflict and confusion. Duplication of effort is also to be avoided. It is hoped that the programs of the two Associations will supplement rather than duplicate the work of each other.

RECOMMENDATIONS*

(1) The Referee concurs in the reports of the Associate Referees on eggs and egg products and on frozen fruits and vegetables.

(2) He also recommends that the subject, canned fish, be broadened to fish and fishery products.

(3) And that work be continued on eggs and egg products, frozen fruits and vegetables, sugar, canned vegetables, canned fruits and other canned acid foods, fish and fishery products, nuts and nut products. Recommendation for future work on canned meats will be made later.

REPORT ON MICROBIOLOGICAL METHODS FOR THE EXAMINATION OF EGGS AND EGG PRODUCTS

By M. T. Bartram (Food and Drug Administration, Federal Security Agency, Washington 25, D.C.), *Associate Referee*

The present method for the examination of eggs and egg products is recorded as first action. At this time certain editorial changes and certain changes which recognize later investigations with these and allied products are necessary.

Research conducted by C. K. Johns, Division of Bacteriology and Dairy Research, Canada, and at present unpublished, has demonstrated that the extent of shaking necessary to disperse the initial dilution can be reduced. This change may be accomplished by rewriting a portion of section **36.3(a)**.

* For report of subcommittee C and action of the Association, see *This Journal*, **36**, 58 (1953).

Similar investigations conducted by the same investigator and by J. B. Hyndman, New Orleans District, Food and Drug Administration, the latter employing dairy and seafood products, have demonstrated the superiority of buffered distilled water as a diluent. Numerous investigators have recognized the toxicity of some distilled waters and many local supplies. Changes in sections **36.3(a)** and **36.11** to recognize the use of buffered diluent are recommended.

Numerous investigations, reported over many years, have demonstrated that incubation at 37° is excessive for most organisms and particularly those occurring in frozen products. All standard procedures such as those recognized by American Public Health Association and U. S. Pharmacopeia have adopted 35°, or lower, rather than 37°. Changes are accordingly recommended in sections **36.5** and **36.7.**

Research culminating in a report by L. Buchbinder, *et al.*, *Public Health Reports,* **66,** 327 (1951), has demonstrated that recognition of media which are chemically better defined and capable of more uniform composition, will not result in significant changes in bacterial results obtained from milk products. Preliminary results confirm this observation with egg products. These media are being adopted by the A.P.H.A. for dairy products including eggs and egg products. Changes in sections **36.4** and **36.10** are accordingly recommended.

Clarifying, editorial, and error correction changes have been made in sections **36.3(b), (c), 36.4, 36.6, 36.7,** and **36.9.** They have been published in *Changes in Methods, This Journal,* **36,** 91 (1953).

RECOMMENDATION

The microbiological method for the examination of eggs and egg products as modified above is recommended for adoption, first action.*

No reports were received on microbiological methods for canned meats, canned acid foods, canned vegetables, and nuts and nut products.

REPORT ON NUTS AND NUT PRODUCTS

By A. M. Henry (Food and Drug Administration, Federal Security Agency, Atlanta, Georgia), *Referee*

The report of the Referee is brief, since unexpected work prevented the preparation and distribution of samples for collaborative work on methods for crude fat, crude protein, ash, reducing sugar, and salt. Also, no work was done on the recommendations for study on sorting methods for moisture, fat, and added starch and oils in peanut butter.

Associate Referee, A. J. Shingler, has reported progress on a study of

* For report of Subcommittee C and action of the Association, see *This Journal,* **36,** 58 (1953).

the methods for added glycerol and propylene glycol in desiccated and canned shredded coconut. His work indicates that cyclohexane as a co-distilling agent will carry over propylene glycol quantitatively, but glycerol only partly. Under present market conditions, glycerol is not used, but sorbitol is added at times. It will be necessary to determine whether sorbitol affects the propylene glycol determination. The Associate Referee expects to complete his study and have a proposed method ready to send out for collaborative study this fall.

Study on chemical methods for decomposition in nuts has been continued. Some work was done in trying out color reactions with fatty acids with the idea that rancid nut meats would develop a distinctive color with dye solutions. None among those tried have been found suitable. Some study was made of the correlation between a rancid taste and the amount of free fatty acids in nut meats. Some correlation was obtained with pecan meats, but a similar correlation could not be obtained with peanut meats. This study will be continued.

It is recommended*—

(1) That methods for moisture, crude fat, crude protein, crude fiber, ash, reducing sugar, and salt be further studied.

(2) That sorting methods for moisture and fat be studied.

(3) That methods for added starch and other additives in peanut butter be studied.

(4) That methods for added propylene glycol, sorbitol, and glycerol in shredded coconut be studied.

(5) That studies on chemical methods for the determination of decomposition in nuts be continued.

No report was received on shredded coconut (glycols and glycerol) or on free fatty acids.

REPORT ON STANDARDIZATION OF MICROCHEMICAL METHODS

By C. O. Willits (Eastern Regional Research Laboratory,† Philadelphia 18, Pennsylvania), *Referee*

The Referees on microchemical methods of analyses have continued their collaborative studies this year. The work on methods for the determination of bromine and chlorine, begun in 1951, showed that the Carius and catalytic methods were equally precise and that they were to be preferred to the Parr bomb method. Based upon the data, details, and

* For report of Subcommittee C and action of the Association, see *This Journal*, 36, 58 (1953).

† One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

modifications of these methods employed by the 1951 collaborators, a tentative procedure was developed for each method, and these have been tested collaboratively this year.

Likewise, the collaborative study of the sulfur method is a continuation of that begun in 1951, in which the collaborative results showed a strong preference for the Carius and catalytic methods, both of which were more precise than the Parr bomb method. This year's study has attempted to determine whether either or both of these preferred methods is sufficiently accurate and precise for adoption as an official method.

Also included in the referee collaborative studies is the Dumas method for nitrogen. Initial work on this method was reported in 1949 in which report it was shown that a minimum temperature of 650°C. was required. An evaluation of the effects of a number of variables in the method still remained to be made. This has been done in the current studies.

The 1952 collaborative studies on microchemical methods have led to the following recommendations*:

(1) That the Carius method for bromine and chlorine as used in the 1952 collaborative studies be adopted, first action.

(2) That further studies of the effect of the temperature of the long burner and of the absorbents in the catalytic combustion method for bromine and chlorine should be made.

(3) That the Carius and catalytic combustion methods as described in the 1952 report on the determination of sulfur be adopted, first action.

(4) That the titrimetric method for determining sulfate formed by either of the two combustion procedures for sulfur be adopted, first action.

(5) That further collaborative work should be done to improve the precision of the sulfur gravimetric method which is required for samples containing phosphorus.

(6) That further collaborative work be done to test the method which will be developed from the results of the 1952 studies of the Dumas procedure.

REPORT ON MICROANALYTICAL DETERMINATIONS OF BROMINE AND CHLORINE

By AL STEYERMARK, *Associate Referee*, and MARIE WALKER GARNER
(Hoffmann-La Roche Inc., Nutley, New Jersey)

Last year's collaborative study (4) on the determination of bromine and chlorine indicated that the Carius and catalytic combustion methods are equally accurate and precise and that each of these is more accurate and

* For report of Subcommittee C and action of the Association, see *This Journal*, 36, 58 (1953).

more precise than the Parr bomb method. Consequently, this year it was decided by the Referees to send out the same two samples, namely bromoacetanilide* and chloroacetanilide,* to each analyst who expressed a willingness to participate in this year's study. These analysts were requested to analyze the samples by a specific procedure which the Referees worked out after consideration of last year's results in which many conditions were studied. It was hoped that by so doing a method would be developed which could be adopted as a first action procedure (either Carius or catalytic combustion or possibly both). With but few exceptions, the collaborators in this year's study adhered fairly closely to the specified procedures for the two methods. Again this year the collaborators were requested to send all of the values obtained so that a statistical analysis of the data could be made to compare the different methods. One collaborator (No. 45) performed a large amount of additional work, in which some of the conditions in the catalytic combustion method were varied, and performed enough analyses so that a separate statistical analysis of his results could be made.

CATALYTIC COMBUSTION METHOD

APPARATUS

(a) *Oxygen supply.*—Use O pressure cylinder with 2 stage reducing valve having needle valve control on low pressure side, or use any other source which will supply pure O at 12 to 15 ml per min.

(b) *Purification train.*—If O is not halogen-free, purify by passing gas through a tube containing first Dehydrite, then Ascarite.

(c) *Combustion tube.*—Quartz (or Vycor) with dimensions shown in Figure 1.

(d) *Absorber.*—Beazley type spiral connected to combustion tube by ground joint.

(e) *Catalyst.*—Two Pt star contacts (5) or two Pt gauze rolls made from 5 cm squares of ca 50 mesh gauze. Rolls to have diam. within one mm of that of combustion tube.

(f) *Furnaces.*—Electric or gas, electric preferred, both providing a temp. inside combustion tube of at least 750°C. and preferably 800°C. or over. Sample burner operated mechanically or manually, the former being preferred. Rate of motion of sample burner: 0.5 cm per min. (20 min. total time for movement).

Rate of oxygen flow: 12 to 15 ml per min.

REAGENTS

(a) *Sodium carbonate solution.*—25 g reagent grade Na_2CO_3 (halogen-free) dissolved in 100 ml boiling distd H_2O.

(b) *Sodium bisulfite solution.* (1)—SO_2 generated by adding concd H_2SO_4 slowly to $NaHSO_3$. The liberated SO_2 is purified by passing it through a tube contg glass wool moistened with halogen-free satd Na_2CO_3 soln. The purified gas is passed into a cooled satd soln of halogen-free Na_2CO_3. The soln so obtained should be stored in sealed ampuls of 3–5 ml capacity, an ampul opened when needed, and again sealed for storage. The prepared soln should conform to the following test: 25 ml of the soln

* The samples were the identically same materials as sent last year, being Eastman Kodak Company materials which were not further purified, since in the Referee's laboratory they were found on analysis to give respective halogen values which differed from the theory by amounts well within the limits of the allowable error (±0.3%) (1, 3).

is made alk. with halogen-free Na_2CO_3 and 3–4 drops 30% H_2O_2 added. The mixture is warmed on the steam bath for 5 min. and cooled. To this is added 1–2 ml halogen-free concd HNO_3 and 0.5 ml 5% $AgNO_3$ soln. After heating for 10 min. on steam bath, no pptn or turbidity should be present.

(c) *Silver nitrate solution.*—5%.

(d) *Nitric acid.*—Reagent grade, sp. gr. 1.42.

SAMPLE

Using a microchemical balance, weigh 5–20 mg sample contg a minimum of 1.5 mg Cl or 2.5 mg Br, or using a semimicrochemical balance, weigh 10–20 mg sample contg a minimum of 2.5 mg Cl or 4.5 mg Br.

Solids, gums, and non-volatile liquids.—Weigh in micro (short or long form) Pt boat.

Volatile liquid.—Weigh in sealed tube 1–2 mm I.D., with capillary end (3). Break off tip of capillary, place both sections in long Pt boat or trough slightly longer than broken tube and insert in combustion tube immediately.

DETERMINATION

Clean catalysts by boiling 10 min. in ca 6 N HNO_3 and flaming over non-luminous flame, using Pt-tipped tweezers. Place catalysts in combustion tube and set tube in furnaces as shown in Figure 1. Heat long furnace to at least 750° and preferably 800°C. or over.

Moisten entire spiral of the absorber by drawing into it with gentle suction 5 ml Na_2CO_3 soln contg 5 drops $NaHSO_3$ soln. Care must be exercised to keep the ground joint dry. Drain excess soln from absorber and attach to combustion tube with ground joint outside of long furnace. Place sample in combustion tube 5 cm from long furnace, connect O source and adjust flow to 12–15 ml per min. using flow meter or calibrated bubble counter.

Heat sample burner to at least 750° and preferably 800°C. or over, bring to 5 cm from sample and move over sample area (10 cm) at rate of 0.5 cm per min. (burning time 20 min.). Continue sweeping with O for 10 min. (total combustion time 30 min.). Disconnect absorber, allow joint to cool (2–3 min.), rinse contents quantitatively into 8-inch test tube with 8–10 ml of distd H_2O contg 2–3 drops of $NaHSO_3$ soln. Add 3 drops 30% H_2O_2 to oxidize excess bisulfite and heat on steam bath 5 min. Cool contents of tube under cold H_2O tap, add 2 ml concd HNO_3 and 2 ml 5% $AgNO_3$ soln. Heat test tube on steam bath, protecting it from light, until ppt is coagulated (ca 1 hr).

Continue detn as directed under Carius method, beginning with "Place previously washed, dried, and weighed filter tube. . . ."

DISCUSSION

Bromine and chlorine results obtained by the catalytic combustion procedure were reported by eight and eleven collaborators respectively. Eight analysts reported 44 values for bromoacetanilide and eleven analysts reported 81 values for chloroacetanilide. The analysts used a catalytic combustion method that was basically the same. In addition, one of the collaborators, No. 45, varied his procedure as to temperature and the absorbent used. A portion of his work was carried out at a long burner temperature of 775°C. and the rest at a long burner temperature of 830°C. Also, part of his determinations were done using sodium carbonate-sodium bisulfite mixture as the absorbent, and part of his results were ob-

tained with potassium hydroxide-hydrazine sulfate as the absorbent. Tables 1 and 2 show the summaries of the analytical data reported for bromoacetanilide and chloroacetanilide, respectively. In these tables n is the number of halogen (bromine and chlorine) values reported by each analyst, $\bar{x}$ is the mean of his data, and s is the standard deviation of his data obtained by the following formula:

$$s = \sqrt{\frac{\Sigma(x - \bar{x})^2}{n - 1}}, \qquad \text{(where } x = \text{ the individual values).}$$

The symbols $\bar{\bar{x}}$ and $s_{\bar{x}}$ are used for the means of all $\bar{x}$'s and the standard deviation of the $\bar{x}$'s, respectively. In the tables are also given the values for $\bar{\bar{x}}$ minus the theory, the mean of the standard deviations, $\bar{s}$, and the mean of the $\bar{x}$ minus the theory. The $\bar{\bar{x}}$'s differ from the theoretical values by -0.19 per cent for bromoacetanilide and -0.20 per cent for chloroacetanilide. The standard deviation of the means, $s_{\bar{x}}$, for the two samples are 0.463 and 0.242. The effect of the variations within the method used by the one collaborator, No. 45, was studied to see if the data indicated that his one procedure would produce more accurate results than his alternates. The two variables that were used by this analyst were the temperature and absorbent, as stated above. The data for the two alternate procedures for the two variables were studied, as shown in Tables 3 and 4, and plotted as shown in Figures 2, 3, and 4. This collaborator, when analyzing bromoacetanilide, performed ten determinations. For six of these he used sodium carbonate-sodium bisulfite as the absorbent and for four others he used potassium hydroxide-hydrazine sulfate, the temperature of the long burner being the same in all cases, namely 830°C. Table 3 and Figure 2 show the effect of using either sodium carbonate-sodium bisulfite or potassium hydroxide-hydrazine sulfate as the absorbent when analyzing bromoacetanilide by the catalytic combustion method. When analyzing chloroacetanilide, this same collaborator performed 34 determinations. In 25 of these, sodium carbonate-sodium bisulfite was used as the absorbent while potassium hydroxide-hydrazine sulfate was used in the other 9. Table 4 and Figure 3 show the effect of this variation in the analysis of chloroacetanilide. Also, during the determinations on chloroacetanilide this collaborator used a long burner temperature of 775°C. for 9 determinations and a temperature of 830°C. for the other 25. Table 4 and Figure 4 show the effect of this in the catalytic combustion of chloroacetanilide.

For each set of data, the Student's t value was calculated using the formula:

$$t = \bar{x}\sqrt{\frac{n_a n_b(n_a + n_b - 2)}{(n_a + n_b)[\Sigma(x_a - \bar{x}_a)^2 + \Sigma(x_b - \bar{x}_b)^2]}},$$

TABLE 1.—*Bromine-catalytic combustion results*

COLLABORATOR NUMBER		1	9	15	28	30	45	51	69	$\bar{\bar{x}}$	$s_{\bar{x}}$
Sample	n	4	6	6	4	8	10	2	4		
Bromoacetanilide	$\bar{x}$	37.51	36.14	37.44	37.28	37.32	36.77	37.31	37.38	37.14	
(37.33% bromine)	s	0.083	0.378	0.311	0.126	0.098	0.458	0.064	0.077		0.463
	$\bar{x}$ − *Theory*	+0.18	−1.19	+0.11	−0.05	−0.01	−0.56	−0.02	+0.05		

$\bar{\bar{x}}$ − *Theory* = −0.19% $\bar{s}$ = 0.199

TABLE 2.—*Chlorine-catalytic combustion results*

COLLABORATOR NUMBER		1	2	9	15	28	30	31	45	51	64	69	$\bar{\bar{x}}$	$s_{\bar{x}}$
Sample	n	6	4	6	6	4	7	4	34	2	4	4		
Chloroacetanilide	$\bar{x}$	20.80	21.06	20.30	20.89	20.75	20.81	20.70	20.35	20.90	20.77	20.44	20.71	
(20.91% chlorine)	s	0.198	0.197	0.190	0.070	0.162	0.044	0.172	0.987	0.014	0.077	0.715		0.242
	$\bar{x}$ − *Theory*	−0.11	+0.15	−0.61	−0.02	−0.16	−0.10	−0.21	−0.56	−0.01	−0.14	−0.47		

$\bar{\bar{x}}$ − *Theory* = −0.20% $\bar{s}$ = 0.257

TABLE 3.—*Effect of variations within the catalytic method for bromine*

Variation		Number of determinations	t	$t_{0.05}$	F	$F_{0.05}$
Absorbent used	Sodium carbonate-sodium bisulfite	6	0.192	2.306	1.334	5.41
	Potassium hydroxide-hydrazine sulfate	4				

Sodium carbonate-sodium bisulfite:

x	$x-$ *Theory*
36.44	−0.89
36.06	−1.27
36.97	−0.36
36.91	−0.42
37.31	−0.02
37.06	−0.27

$\bar{x}=36.79\%$ $\bar{x}-$ *Theory* $=-0.54$

Potassium hydroxide-hydrazine sulfate:

x	$x-$ *Theory*
35.96	−1.37
36.80	−0.53
37.06	−0.27
37.09	−0.24

$\bar{x}=36.73\%$ $\bar{x}-$ *Theory* $=-0.60$

TABLE 4.—*Effect of variations within the catalytic method for chlorine*

Variation		Number of determinations	t	$t_{0.05}$	F	$F_{0.05}$
Absorbent used	Sodium carbonate-sodium bisulfite	25	1.07	2.04	7.714	3.12
	Potassium hydroxide-hydrazine sulfate	9				
Temperature of long burner	775°C.	9	1.802	2.04	4.884	2.36
	830°C.	25				

Sodium carbonate-sodium bisulfite

x	$x-$ *Theory*
20.70	−0.21
19.99	−0.92
20.71	−0.20
19.53	−1.38
19.92	−0.99
20.72	−0.19
20.68	−0.23
16.02	−4.89
20.50	−0.41
20.68	−0.23
22.07	+1.16
18.72	−2.19
19.48	−1.43
20.16	−0.75
20.70	−0.21
20.53	−0.38
20.35	−0.56
19.33	−1.58
20.83	−0.08
20.97	+0.06
20.70	−0.21
19.91	−1.00
20.83	−0.08
21.12	+0.21
20.96	+0.05

$\bar{x}=20.24\%$
$\bar{x}-$ *Theory* $=-0.67$

Potassium hydroxide-hydrazine sulfate

x	$x-$ *Theory*
20.58	−0.33
20.60	−0.31
20.60	−0.31
21.10	+0.19
20.57	−0.34
20.68	−0.23
19.78	−1.13
20.72	−0.19
21.20	+0.29

$\bar{x}=20.65\%$
$\bar{x}-$ *Theory* $=-0.26$

775°C.

x	$x-$ *Theory*
20.70	−0.21
19.99	−0.92
20.71	−0.20
19.53	−1.38
19.92	−0.99
20.72	−0.19
20.68	−0.23
16.02	−4.89
20.50	−0.41

$\bar{x}=19.86\%$
$\bar{x}-$ *Theory* $=-1.05$

830°C.

x	$x-$ *Theory*
20.68	−0.23
22.07	+1.16
18.72	−2.19
19.48	−1.43
20.16	−0.75
20.70	−0.21
20.58	−0.33
20.60	−0.31
20.60	−0.31
21.10	+0.19
20.57	−0.34
20.68	−0.23
20.53	−0.38
19.78	−1.13
19.33	−1.58
20.83	−0.08
20.97	+0.06
20.72	−0.19
20.70	−0.21
19.91	−1.00
21.20	+0.29
20.35	−0.56
20.83	−0.08
21.12	+0.21
20.96	+0.05

$\bar{x}=20.53\%$
$\bar{x}-$ *Theory* $=-0.38$

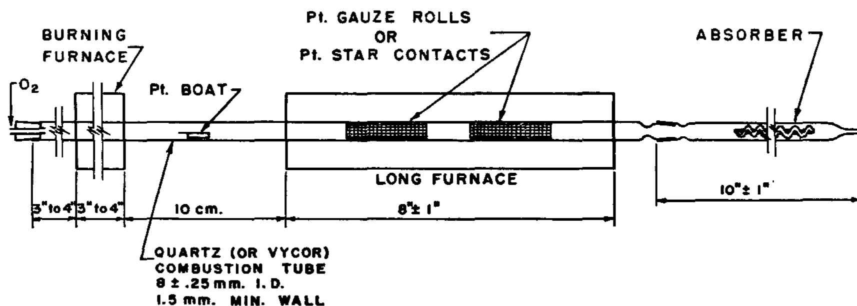

FIG. 1.—Combustion Assembly for Halogen Determination by Catalytic Method.

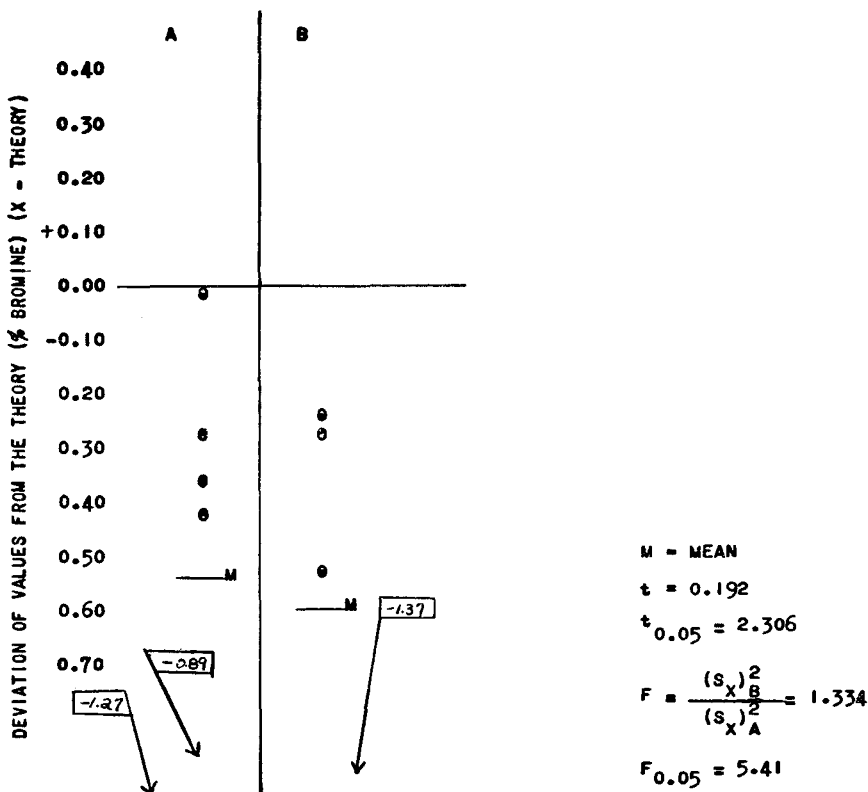

FIG. 2.—Variation of Catalytic Method for Bromine (absorbent) (Collaborator #45). Sodium Carbonate-Sodium Bisulfite (A) vs Potassium Hydroxide-Hydrazine Sulfate (B).

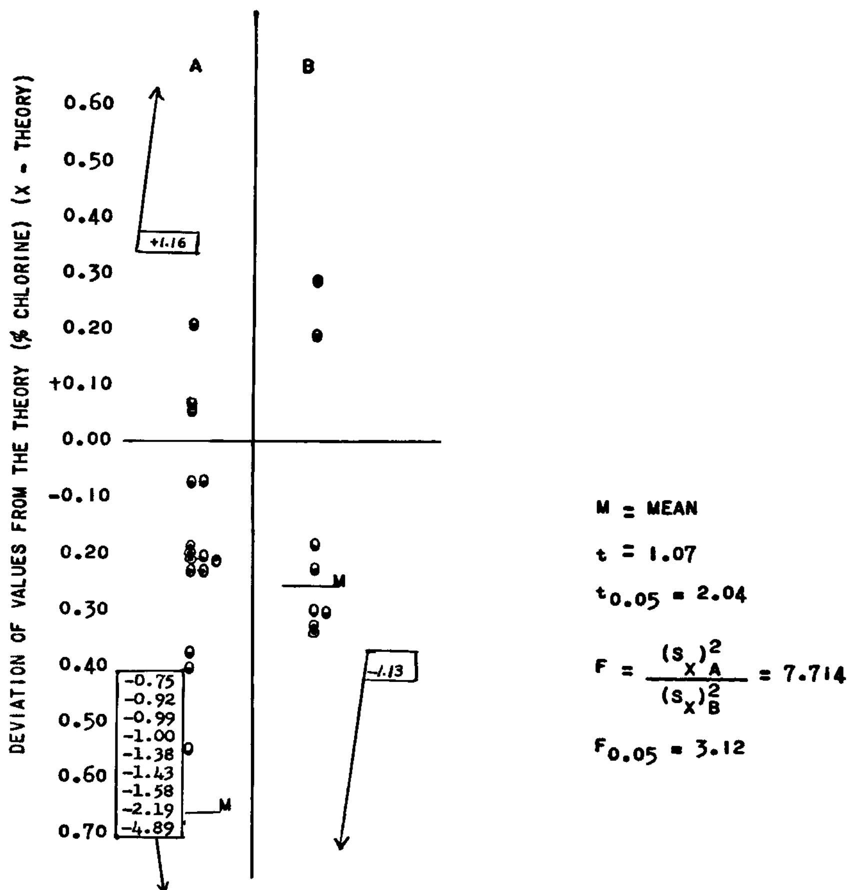

FIG. 3.—Variation of Catalytic Method for Chlorine (absorbent) (Collaborator #45). Sodium Carbonate-Sodium Bisulfite (A) vs Potassium Hydroxide-Hydrazine Sulfate (B).

where $\bar{x} = \bar{x}_a - \bar{x}_b$; n_a and n_b are the number of values in groups a and b, respectively; x_a and x_b are individual values for the two groups, $\bar{x}_a$ and $\bar{x}_b$, the means of the values for the two groups. If the calculated t value was greater than the critical value ($t_{0.05}$) obtained from a table of Student's t's (2, 7), the difference between the two means was significant at the 5 per cent level, and the procedure whose mean was nearer the theoretical value was considered to be the better. If the t value was not greater than the critical value, the variance ratio F value was calculated by the equation:

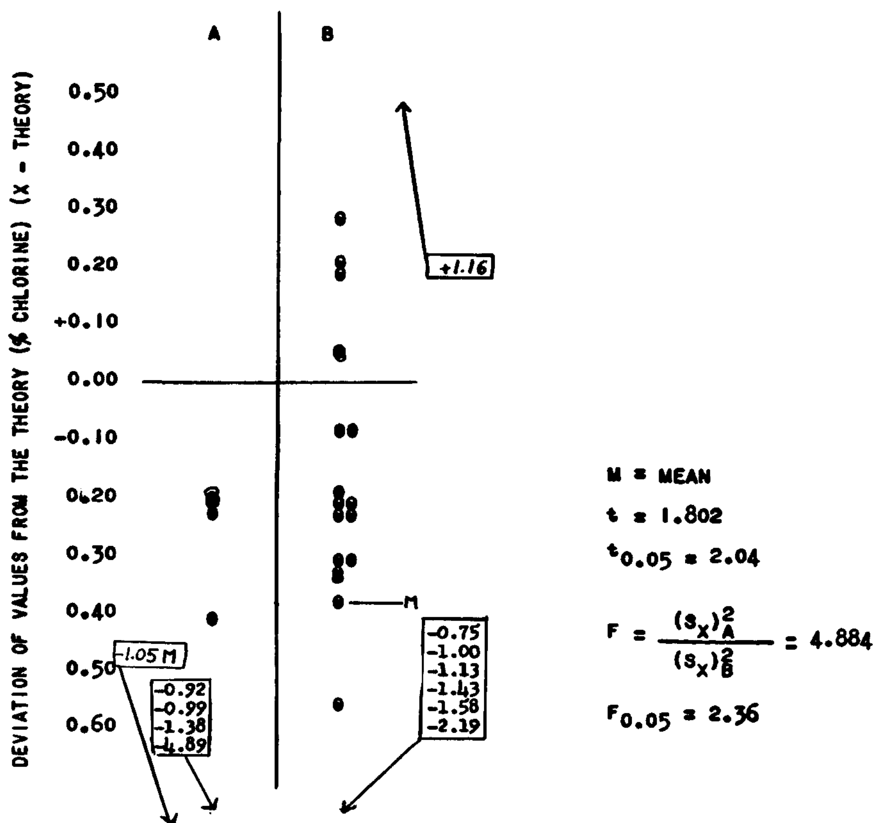

FIG. 4.—Variations of Catalytic Method for Chlorine (temperature) (Collaborator #45). 775°C. (A) vs 830°C. (B).

$$F = \frac{(s_{\bar{x}})_a^2}{(s_{\bar{x}})_b^2}$$

where $(s_{\bar{x}})_a^2$ is always the larger value. If the calculated F value was greater than the critical value ($F_{0.05}$) obtained from the table of F values (2, 7) the difference in precision between the two groups of data was significant at the 10 per cent level and the procedure with the lower $s_{\bar{x}}$ was the more precise. In the comparison of the values obtained for bromoacetanilide with the catalytic combustion method, using either sodium carbonate-sodium bisulfite or potassium hydroxide-hydrazine sulfate as the absorbent, as shown in Table 3 and Figure 2, they did not yield either t or F values which were critical ($t = 0.192$ and $t_{0.05} = 2.306$; $F = 1.334$

and $F_{0.05}=5.41$). However, when the absorbent was varied in the analysis of chloroacetanilide by the catalytic combustion method, the data of which are shown in Table 4 and Figure 3, the t value was not found to be critical ($t=1.07$ and $t_{0.05}=2.04$), but the F value was critical ($F=7.714$ and $F_{0.05}=3.12$) and in favor of the use of potassium hydroxide-hydrazine sulfate. A comparison of the data obtained for chloroacetanilide, in which the temperature of the catalytic combustion was varied, is shown in Table 4 and Figure 4. In Figure 4 under the B column, the results obtained at a long burner temperature of 830°C. are shown, while under the A column, those obtained at 775°C. are shown. Upon calculation of the t value it was found to be not critical ($t=1.802$ while $t_{0.05}=2.04$). However, calculation of the F value showed that the temperature was critical and in favor of 830°C. ($F=4.884$ and $F_{0.05}=2.36$).

CARIUS COMBUSTION METHOD*

REAGENTS

(a) *Fuming nitric acid.*—Reagent grade, halogen-free, sp. gr. 1.50.
(b) *Silver nitrate.*—Reagent grade, powd.

APPARATUS

(a) *Combustion tubes.*—Use clean, 240 ± 10 mm long by 13 ± 0.7 mm O.D. standard wall Pyrex tubes or 210 ± 10 mm long by 13 ± 0.8 mm O.D. Pyrex tubes with 2.3 ± 0.3 mm walls (see Table 5), free from flaws and with a rounded seal at the bottom (see Figure 5) (3, 6).

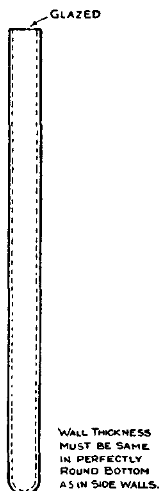

FIG. 5.—Combustion Tube.

(b) *Furnace.*—Elec. with capacity of 4 or more tubes held at an angle of ca 45°. Furnace must maintain temp. of 250 ± 10 or 300 ± 10°C. for 5 or more hrs, with no more than 5°C. difference between any 2 points on a tube or 5° difference between similar points on any 2 tubes. Furnace must have variable resistor or other device to adjust furnace to desired temp. Open end of furnace wells must have safety device to retain glass in furnace should tube explode, and device must be provided for removing individual tubes from wells (6).

(c) *Filter tubes.*—Micro filter tube with medium coarse porosity (av. pore diam. 15–25 μ), fritted disc and capacity of 3 ml (5).

SAMPLE

Using a microchemical balance, weigh 5–20 mg sample contg a min. of 1.5 mg Cl or 2.5 mg Br; or using a semimicrochemical balance, weigh 10–20 mg sample contg a min. of 2.5 mg Cl or 4.5 mg Br.

Solid samples.—Weigh by difference in charging tube (5).

Viscous liquids or gummy solids.—Weigh in porcelain boat (3).

Volatile liquids.—Weigh in 5 cm sealed glass tube, 1–2 cm I.D. with capillary tip (3). Break off tip of the capillary before placing in combustion tube sealed end down.

* To alter the conditions (temp., size of sample, vol. of acid, etc.) might prove to be dangerous, presenting an explosion hazard.

DETERMINATION

Place weighed sample in combustion tube, add powd. $AgNO_3$ 100% in excess of amount estimated to be necessary, and add 0.3 ± 0.03 or 0.5 ± 0.05 ml fuming HNO_3 depending on type of combustion tube (see Table 5). Using blast lamp and holding tube at 30–40° angle, seal tube at a distance from bottom so that sealed tube will have length shown in Table 5. Rotate tube slowly in flame until wall thickens, pull out and seal off narrow neck of tube (1, 3). Wall of seal should not be less than ¾ the thickness of tube wall. (If sample and HNO_3 react at room temp., immediately cool bottom of tube in ice H_2O or dry ice-acetone bath.) Immediately place tube in furnace. Heat tubes for 5 hrs at 250 or 300 ± 10°C. (see Table 5) (3, 6).

Observe the following precautions before and during opening of combustion tubes: (a) Place asbestos glove on hand used to hold small burner or hand torch; (b) protect face by transparent face mask or work behind safety shield; (c) be certain tube has cooled to room temp.; (d) force tip of tube ca 2″ out of furnace well; (e) gently flame end to drive all acid from tip and upper walls; and (f) soften tip with small hot flame until pressure in tube is released by blowing out softened glass (3).

Remove vented tube from furnace and cut off constricted end by scratching tube with file ca ½″ from shoulder of open end, moisten scratch, and touch with tip of very hot glass rod. Remove end of the tube with care and fire polish to avoid contaminating ppt with glass splinters.

Rinse walls of tube with distd H_2O until tube is ca ¾ full, place in steam or boiling H_2O bath, protected from light, and digest until ppt is coagulated (ca 30 min.). Place previously washed, dried, and weighed filter tube in one-holed stopper in suction flask, connect short arm of siphon tube to filter tube thru small rubber stopper and adjust Carius tube so that long arm of siphon almost touches the ppt (1, 3, 5). Transfer ppt to filter tube by suction. Rinse tube and ppt alternately with 1% HNO_3 and 95% ethanol using 2 or 3 ml portions for each rinse.

Remove siphon, rinse tip and stopper with alcohol and rinse filter tube and ppt first with acid, then with alcohol. Wipe outside of filter tube with moist chamois (or cheesecloth) and dry at 125°C. for 30 min. in air oven or 80°C. for 30 min. in vacuum oven, cool to room temp. (30 min.), and weigh. Handle dry tube with chamois finger cots or tweezers. Make blank run and subtract any correction from wt of sample ppt.

CALCULATIONS

$$\frac{(\text{wt ppt} - \text{blank}) \times \frac{\text{Cl}}{\text{AgCl}}}{\text{wt sample}} \times 100 = \%\ \text{Cl}$$

$$\frac{(\text{wt ppt} - \text{blank}) \times \frac{\text{Br}}{\text{AgBr}}}{\text{wt sample}} \times 100 = \%\ \text{Br}$$

DISCUSSION

Bromine and chlorine results obtained by the Carius combustion procedure were reported by eleven and ten collaborators respectively. Eleven analysts reported 74 values for bromoacetanilide and ten analysts reported 71 values for chloroacetanilide. All of the procedures were basically the same. Tables 6 and 7 show summaries of the analytical data reported. In these tables the symbols used are the same as those used in Tables

TABLE 5.—*Combustion tubes*

COMBUSTION TUBE	WALL THICKNESS MM	O.D. MM	LENGTH MM	LENGTH OF SEALED TUBE BETWEEN BOTTOM AND START OF TAPER AT SHOULDER MM	VOL. HNO_3 (SP. GR. 60°F., APPROXIMATELY 1.5) ML	TEMP. °C.
Heavy-walled	2.3±0.3	13±0.8	210±10	150 to 175	0.5	250
Thin-walled	1.2±0.2	13±0.7	240±10	180 to 210	0.3	300

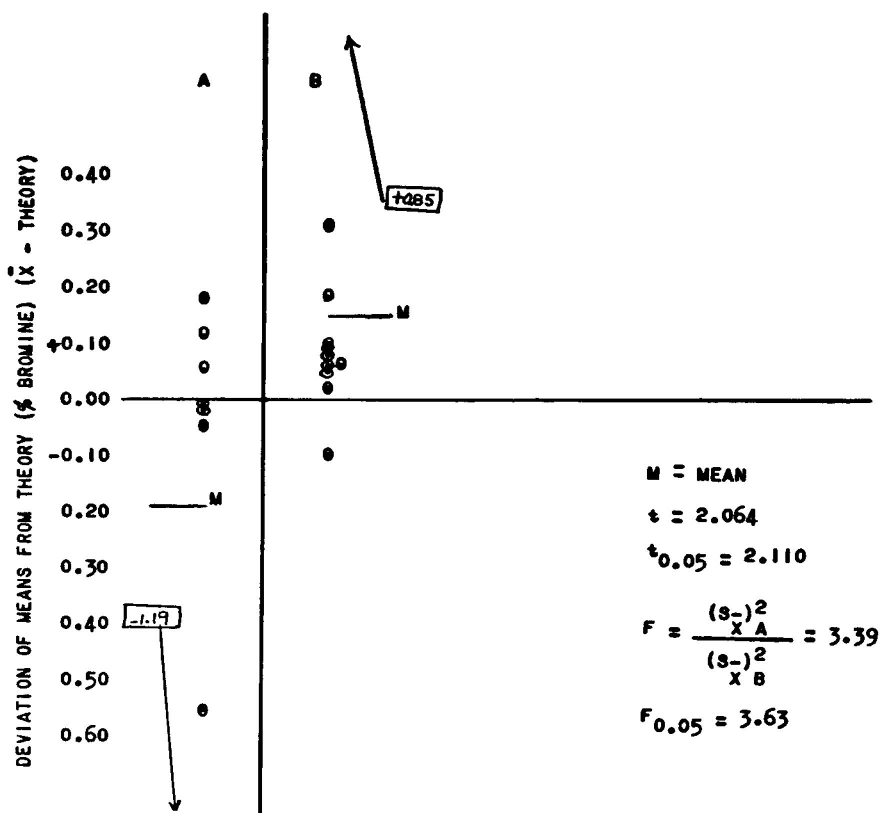

FIG. 6.—Determination of Bromine. Catalytic Method (A) vs Carius Method (B).

TABLE 6.—*Bromine-Carius combustion results*

COLLABORATOR NUMBER		0	15	22	29	31	37	49	50	57	62	63	$\bar{\bar{x}}$	$s_{\bar{x}}$
Sample	n	8	4	4	5	4	4	13	6	5	16	4		
Bromacetanilide	$\bar{x}$	37.35	37.43	37.41	38.18	37.42	37.64	37.51	37.38	37.23	37.39	37.39	37.48	
(37.33% bromine)	s	0.103	0.088	0.056	0.687	0.063	0.223	0.240	0.129	0.040	0.220	0.083		0.252
	$\bar{x}$ − *Theory*	+0.02	+0.10	+0.08	+0.85	+0.09	+0.31	+0.18	+0.05	−0.10	+0.06	+0.06		

$\bar{\bar{x}}$ − *Theory* = +0.15% $\bar{s}$ = 0.176

TABLE 7.—*Chlorine-Carius combustion results*

COLLABORATOR NUMBER		0	15	22	29	37	49	50	57	62	63	$\bar{\bar{x}}$	$s_{\bar{x}}$
Sample	n	8	4	4	6	5	12	6	4	18	4		
Chloroacetanilide	$\bar{x}$	20.84	20.91	20.80	21.11	20.79	20.94	20.80	20.81	20.98	20.81	20.88	
(20.91% chlorine)	s	0.141	0.084	0.141	0.230	0.498	0.144	0.141	0.048	0.231	0.048		0.105
	$\bar{x}$ − *Theory*	−0.07	0.00	−0.11	+0.20	−0.12	+0.03	−0.11	−0.10	+0.07	−0.10		

$\bar{\bar{x}}$ − *Theory* = −0.03% $\bar{s}$ = 0.171

1 and 2. The $\bar{x}$'s differ from the theoretical values by only +0.15% for bromoacetanilide and −0.03% for chloroacetanilide. There was practically no difference between the standard deviations of the mean, $s_{\bar{x}}$, which were 0.176 and 0.171 respectively.

COMPARISON OF METHODS, CARIUS vs CATALYTIC COMBUSTION

A comparison was made between these two methods to see whether or not either gave more accurate or precise results than the other. These results are shown in Table 8 and Figures 6 and 7. Figure 6 shows the comparison between the two methods in the analysis of bromoacetanilide and Figure 7 shows the comparison of the results obtained in the analysis of chloroacetanilide. For bromoacetanilide the comparison gave $t=2.064$ while $t_{0.05}=2.110$. Likewise, calculation of the F value did not yield a critical figure ($F=3.39$ and $F_{0.05}=3.63$). Comparison of the two methods used for chloroacetanilide, shown in Figure 7, gave $t=2.050$ while $t_{0.05}=2.093$. Calculation of the F value, however, showed that the difference in precision is critical and in favor of the Carius method ($F=5.27$ and

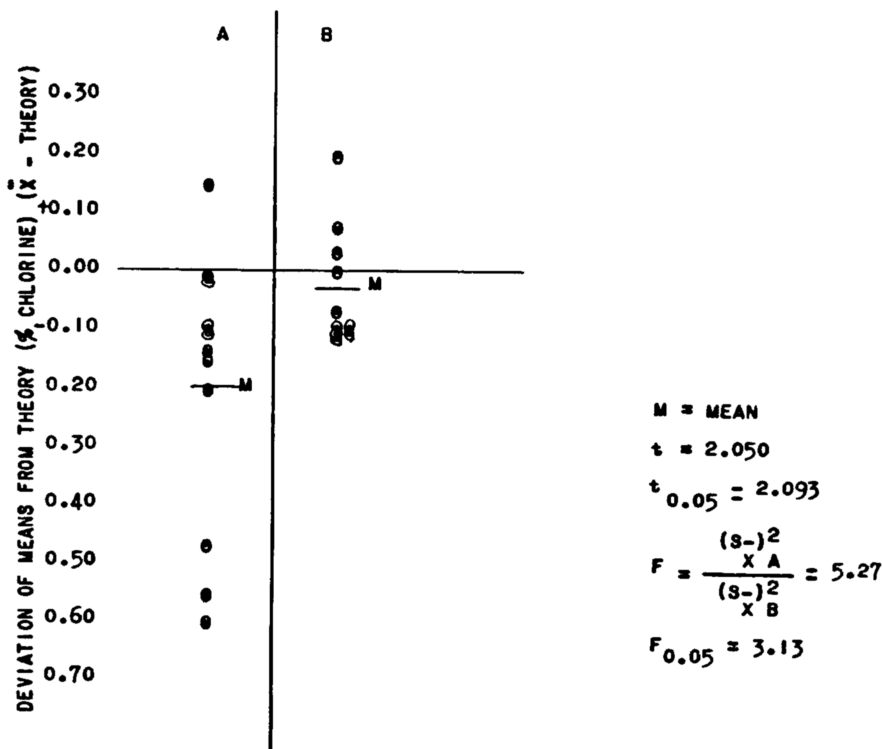

FIG. 7.—Determination of Chlorine. Catalytic Method (A) vs Carius Method (B).

TABLE 8.—*Comparison of methods*

DETERMINATION OF	COMPARISON OF	NUMBER OF COLLABORATORS	t	$t_{0.05}$	F	$F_{0.05}$
Bromine	Carius	11	2.064	2.110	3.39	3.63
	Catalytic	8				
Chlorine	Carius	10	2.050	2.093	5.27	3.13
	Catalytic	11				

$F_{0.05}=3.13$). Although the t values for both bromoacetanilide and chloroacetanilide are not critical at the 5 per cent level, they are all critical at the 10 per cent level.

COMPARISON OF RESULTS, 1951 vs 1952

Table 9 shows the comparison of the results obtained on both bromoacetanilide and chloroacetanilide when analyzed by both the Carius and catalytic combustion methods. In this table are shown the $\bar{\bar{x}}$ values, the $\bar{\bar{x}}-$theory, the $s_{\bar{x}}$ and the $\bar{s}$ values. The symbols used are the same as those used in the other tables. The results on bromoacetanilide, analyzed by both the Carius and catalytic combustion methods, were slightly better during 1951 than in 1952 (as shown by the $\bar{\bar{x}}-$theory, $s_{\bar{x}}$, and $\bar{s}$ values). The results for chloroacetanilide by the Carius method were essentially the same as in 1951; the results obtained with the catalytic combustion method were slightly better during 1951 than during 1952 (as shown by the $\bar{\bar{x}}-$theory, $s_{\bar{x}}$, and $\bar{s}$ values).

CONCLUSIONS

1. *Determination of bromine; Carius versus catalytic combustion method.*— Calculation of the t and F values did not prove critical at the 5 per cent level, but were critical at the 10 per cent level and in favor of the Carius method.

2. *Determination of chlorine; Carius versus catalytic combustion method.*—

TABLE 9.—*Comparison of results for 1951 and 1952*

COMPOUND	METHOD	YEAR	THEORY	$\bar{\bar{x}}$	$\bar{\bar{x}}$ − THEORY	$s_{\bar{x}}$	$\bar{s}$
Bromo-acetanilide	Carius	1951	37.33% Bromine	37.35%	+0.02	0.187	0.225
		1952	37.33% Bromine	37.48%	+0.15	0.252	0.176
	Catalytic	1951	37.33% Bromine	37.38%	+0.05	0.114	0.181
		1952	37.33% Bromine	37.14%	−0.19	0.463	0.199
Chloro-acetanilide	Carius	1951	20.91% Chlorine	20.84%	−0.07	0.139	0.198
		1952	20.91% Chlorine	20.88%	−0.03	0.105	0.171
	Catalytic	1951	20.91% Chlorine	20.89%	−0.02	0.158	0.185
		1952	20.91% Chlorine	20.71%	−0.20	0.242	0.257

The calculated t value was not critical at the 5 per cent level, but was at the 10 per cent level. The calculated F value was critical at the 10 per cent level, and both were in favor of the Carius method.

3. *Determination of chlorine by the catalytic combustion method; potassium hydroxide-hydrazine sulfate versus sodium carbonate-sodium bisulfite used as the absorbing agent.**—Comparison did not give a critical t value, but calculation of the F value showed that the difference in precision is significant and in favor of potassium hydroxide-hydrazine sulfate.

4. *Determination of chlorine by catalytic combustion method; temperature of long burner 830°C. versus 775°C.**—Comparison did not give a critical t value, but calculation of the F value showed that the difference in precision is significant and in favor of 830°C.

RECOMMENDATIONS

The Associate Referee recommends that:

1. The Carius method for bromine and chlorine be adopted as official, first action.

2. Further work be done to study the effect of the temperature of the long burner and of the absorbents in the catalytic combustion method.

ACKNOWLEDGMENT

The authors are indebted to Eleanor E. Loeschauer for the preparation of Figures 2, 3, 4, 6, and 7. Figure 5 is published through the courtesy of *Analytical Chemistry*.

COLLABORATORS

The collaborators on the bromine and chlorine analyses were:

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* Work of collaborator No. 45.
† For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 58 (1953).

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REPORT ON MICROANALYTICAL DETERMINATION OF SULFUR

By C. L. OGG (Eastern Regional Research Laboratory, ARA, U. S. Dept. of Agriculture, Philadelphia 18, Pa.), *Associate Referee*

Last year's collaborative studies (1) on the determination of sulfur indicated a preference for the Carius and catalytic combustion procedures over the Parr bomb method, both from the data obtained last year and also from the relative number of analysts using the Parr bomb procedure. Consequently, the objective of this year's work was to test the two preferred procedures, the Carius and the catalytic combustion, to see if they were sufficiently accurate and precise to warrant recommending them for adoption, first action. The details of the procedures were based upon the results of the statistical study of last year's data. In those instances where the results showed no preference for one technique or condition over another, the condition or technique included in this year's method was either the one most frequently used by the collaborators, the more simple technique, or the more easily attained condition. The same two samples, namely, benzyl-isothiourea hydrochloride and sulfanilamide, were sent to each collaborator with copies of the tentative procedures, which they were requested to follow as closely as possible. With but few exceptions, the collaborators adhered closely to the specified procedures. Three analysts used the one-piece Pregl combustion tube instead of the two-piece Beazley type and two used a Grote absorber instead of the spiral, but in no case was the change believed to be important, because neither of these variations affected the results significantly in last year's

study. Collaborator 2 used a more rapid oxygen flow rate in the catalytic combustion than was specified but used the Grote absorber to counteract this. He also used a photometer rather than a visual comparator to determine the end point in the titration of the sulfate. Collaborator 30 used an electroprecipitator instead of bromine for absorbing the sulfur trioxide. These variations had little or no effect on the results; therefore their data was used in the statistical calculations.

The procedures for the Carius and the catalytic combustion methods for the micro determination of sulfur which were sent to the collaborators for study this year were as follows:

CARIUS COMBUSTION METHOD

REAGENTS

(a) *Fuming nitric acid.*—Reagent grade, sp. gr. 1.50.

(b) *Sodium chloride.*—Reagent grade, fine crystals.

For volumetric determination:

(c) *Barium chloride solution.*—Ca 0.02 *N*, standardized by titrating 5–7 mg freshly dried K_2SO_4, A.C.S. specification (weighed to the nearest 0.01 mg) by same procedure used for the sample titration. Correct titration for indicator error by blank run.

(d) *Potassium sulfate.*—A.C.S., powd. and dried.

(e) *Sodium hydroxide.*—Ca 0.1 *N*.

(f) *Hydrochloric acid.*—Ca 0.02 *N*.

(g) *Phenolphthalein.*—0.5% soln in 50% alcohol.

(h) *Sulfate indicator.*—"T.H.Q." prepd indicator (Betz Laboratories, Philadelphia, Pa.).

For gravimetric determination:

(i) *Dilute hydrochloric acid.*—Add 1 ml concd HCl to 300 ml distd H_2O.

(j) *Barium chloride solution.*—10%.

(k) *Barium chloride solution.*—Ca 0.1 *N* (for alternate grav. detn.).

APPARATUS

(a) *Combustion tubes.*—Use clean, 240±10 mm by 13±0.7 mm O.D. standard wall pyrex glass tubes or 210±10 mm by 13±0.8 mm O.D. pyrex glass tubes with 2.3±0.3 mm walls (see Table 1) free from flaws and with rounded seal at the bottom.

(b) *Furnace.*—Elec. with capacity of 4 or more tubes held at angle of ca 45°. Furnaces must maintain temp. of 250±10 or 300±10°C. for 5 or more hr, with no more than 5° difference between any two points on a tube, or 5° difference between similar points on any two tubes. Furnace must have variable resistor or other device to adjust furnace to desired temp. Open end of furnace wells must have safety device to retain glass in furnace should tube explode, and device must be provided for removing individual tubes from wells.

(c) *Crucible and filter stick* (for gravimetric determination).—Porcelain crucible, ca 15 ml capacity, with black inside glaze weighing about 10 g. Porcelain filter stick with unglazed bottom, weighing about 2 g (3).

(d) *Filter tubes* (for alternate gravimetric determination).—Micro filter tube with medium porosity fritted disc and capacity of 3 ml.

(e) *Titration assembly.*—5 ml buret graduated in 0.01 ml; rectangular titration

cell ca 2×4×5 cm with min. capacity of 50 ml; and standard orange-red glass color filter having 37% transmittancy at 550 mμ. (Arthur H. Thomas Co., Philadelphia, Pa., Cat. No. 9324-H.) Cell and filter are placed side by side on milk glass window illuminated from below, preferably by fluorescent light. The light source must be masked so that only the cells and filter are illuminated.

SAMPLE

Using microchemical balance, weigh 5–20 mg sample contg not less than 0.75 mg S or using semimicrochemical balance, weigh 10–20 mg sample contg not less than 0.75 mg S for volumetric analysis, or 1.5 mg S if gravimetric procedure is to be used.

Solid samples.—Weigh by difference in charging tube.

Viscous liquids and gummy solids.—Weigh in micro porcelain boat.

Volatile liquids.—Weigh in ca 5 cm long, 1–2 mm I.D. sealed glass tube with capillary tip. Break off tip of capillary before placing in combustion tube sealed end down.

DETERMINATION

Place weighed sample in combustion tube, add NaCl 100% in excess of amount equivalent to S in sample, and add 0.3 ±0.03 or 0.5 ±0.05 ml fuming HNO_3 depending on type of combustion tube (see Table 1). Using blast lamp and holding tube at 30–40° angle, seal tube at a distance from bottom so that sealed tube will have

TABLE 1.—*Combustion tubes*

COMBUSTION TUBE	WALL THICKNESS	O.D.	LENGTH	LENGTH OF SEALED TUBE BETWEEN BOTTOM AND START OF TAPER AT SHOULDER	VOL. HNO_3 (SP. GR. AT 60°F., APPROXIMATELY 1.5)	TEMP. °C.
	mm	*mm*	*mm*	*mm*	*ml*	
Heavy-walled	2.3 ±0.3	13 ±0.8	210 ±10	150 to 175	0.5	250
Thin-walled	1.2 ±0.2	13 ±0.7	240 ±10	180 to 210	0.3	300

length shown in Table 1. Rotate tube slowly in flame until wall thickens, pull out and seal off narrow neck of tube. Wall of seal should not be less than ¾ the thickness of tube wall. (If sample and nitric acid react at room temp., immediately cool bottom of tube in ice-H_2O or dry ice-acetone bath.) Immediately place tube in furnace. Heat tube for 5 hr at 250 or 300 ±10°C. (see Table 1).

Observe the following precautions before and during opening of combustion tubes. (a) Place asbestos glove on hand used to hold small burner or hand torch; (b) protect the face by transparent face mask or work behind safety shield; (c) be certain tube has cooled to room temp.; (d) force tip of tube about 2 inches out of furnace well; (e) gently flame end to drive all acid from tip and upper walls; and (f) soften tip with small hot flame until pressure in tube is released by blowing out softened glass.

Remove vented tube from the furnace and cut off constricted end by scratching tube with file 0.5–1″ below shoulder of open end, moisten scratch, and touch with tip of very hot glass rod. Remove end of tube with care and fire polish if gravimetric procedure is to be used to avoid contaminating ppt. with glass splinters. Transfer

contents of tube to 50 ml beaker, rinsing tube 4–6 times with 3–5 ml H_2O. Evap. to dryness on steam bath.

*Volumetric determination.**—Dissolve residue in 10 ml distd H_2O. Pour soln into titration cell, add one drop phenolphthalein indicator, make just alk. with 0.1 *N* NaOH, then acid with 0.02 *N* HCl adding one drop in excess. Add ca 0.15 g of "T.H.Q." indicator, stir to dissolve, rinse beaker 2 or 3 times using sufficient ethanol so that final soln is ca 50% ethanol. Titrate with standard $BaCl_2$ soln from 5 ml burette graduated in 0.01 ml until stable color of the soln immediately after stirring matches standard glass color filter. (Make certain end point taken is real and not pseudo end point which will fade on standing 1–2 min.) Run blank on reagents and correct titration value.

CALCULATION

$$\frac{(\text{ml } BaCl_2 - \text{blank}) \times N \times 16.033 \times 100}{\text{Sample wt (mg)}} = \% \, S$$

Gravimetric determination.—Dissolve residue in 3–5 ml of 1:300 HCl, pour into previously ignited and weighed porcelain crucible. Rinse beaker with four 1–2 ml portions of 1:300 HCl and place crucible on steam bath until soln is near boiling point. (If total vol. exceeds 10–11 ml evap. to this vol.) Add 1 ml 10% $BaCl_2$, digest for at least 0.5 hr and cool to room temp. Connect porcelain filter stick previously ignited and weighed with crucible to arm of siphon with rubber tubing, the other arm of siphon being connected to suction flask through a rubber stopper. Lower filter stick into crucible, draw off soln, rinse ppt., walls of crucible, and filter stick alternately with 1–2 ml portions of 1:300 HCl and ethanol, drawing off as much liquid as possible. Carefully detach filter stick, place in crucible and wipe outside of crucible with moist chamois or cheesecloth. Dry in oven at ca 110°C. for 10 min., then ignite in muffle furnace at 700°C. for 10 min. (Ignition may be carried out by setting crucible containing filter stick in larger porcelain crucible and heating larger crucible to dull red heat with Meker burner.) Cool on metal block for 20 min. (or in desiccator for 1 hr) and weigh. Make blank run on reagents.

CALCULATION

$$\frac{(\text{wt } BaSO_4 - \text{blank}) \times \frac{S}{BaSO_4} \times 100}{\text{Sample wt}} = \% \, S$$

Alternate gravimetric determination.—Dissolve residue in 10 ml distd H_2O. Add 1 drop concd HCl, heat to near 90°C. on steam bath, add 5 ml 0.1 *N* $BaCl_2$, digest for at least ½ hr and cool to room temp. Place previously washed, dried, and weighed filter tube (medium porosity) in one-hole stopper in suction flask, connect small funnel (1–1.5" diam.) to filter through small rubber stopper with funnel tip protruding ca 0.25". Transfer ppt through funnel to filter tube, rinsing beaker and funnel alternately with 1:300 HCl and 95% ethanol using 2 or 3 ml portions. Remove funnel, rinse tip and stopper with alcohol, then rinse filter and ppt with 1:300 HCl, then with alcohol. Wipe outside of filter tube with moist chamois (or cheesecloth) and dry either at 135°C. in air oven or at 80° in vacuum oven for 0.5 hr, place in open air by balance until cooled to room temperature (20 min.) and weigh. Handle dry tube with clean chamois finger cots or tweezers. Make blank run and subtract any correction from weight of sample ppt.

* Volumetric detn. cannot be used if sample contains phosphorus.

CALCULATION

$$\frac{(\text{wt BaSO}_4 - \text{blank}) \times \frac{S}{\text{BaSO}_4} \times 100}{\text{Sample wt}} = \% \, S$$

CATALYTIC COMBUSTION METHOD

REAGENTS

Use reagents of the Carius combustion method above, and

(a) *Bromine water*.—Satd aq. soln of Br stored in glass-stoppered bottle.

For gravimetric determination:

(b) *Hydrogen peroxide solution*.—Dil. 20 ml reagent grade 30% H_2O_2 with 80 ml distd H_2O.

APPARATUS

(a) *Oxygen supply*.—Use O pressure cylinder with 2 stage reducing valve having needle valve control on low pressure side, or any other source which will supply pure O at 12–15 ml/min.

(b) *Purification train*.—If O is not free from S-contg gases, purify by passing gas through a tube containing first Dehydrite, then Ascarite.

(c) *Combustion tube*.—Quartz (or Vycor) with dimensions shown in Figure 1.

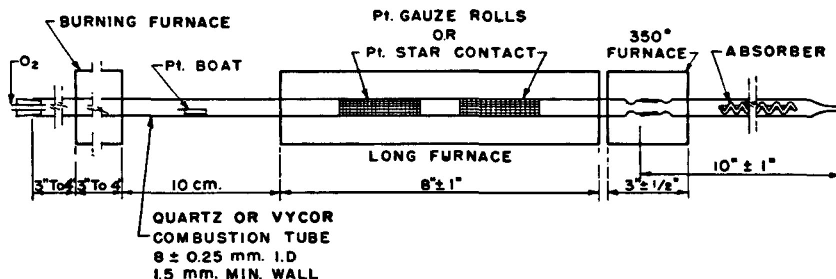

FIG. 1.—Combustion Assembly for Sulfur Determination by Catalytic Method.

(d) *Absorber*.—Beasley type spiral connected to combustion tube by ground joint.

(e) *Catalyst*.—2 Pt star contacts (3) or 2 Pt gauze rolls made from 5 cm squares of ca 50 mesh gauze. Rolls to have diam. within 1 mm of I.D. of combustion tube.

(f) *Furnaces*.—Elec. or gas, elec. preferred, both providing temp. inside combustion tube of at least 750°C. and preferably 800°C. or over. Sample burner operated mechanically or manually, the former being preferred. Short furnace for ground joint, preferably elec., to operate at ca 350°C. Rate of motion of sample burner 0.5 cm/min.

(g) *Titration assembly*.—See Carius combustion method above.

(h) *Crucible and filter stick* (*for gravimetric determination*).—Porcelain crucible, ca 15 ml capacity, with black inside glaze, weighing about 10 g. Porcelain filter stick, with unglazed bottom, weighing about 2 g (3).

(i) *Filter tubes* (*for gravimetric determination*).—Micro filter tube with medium porosity fritted disc and capacity of 3 ml.

SAMPLE

Use sample described under Carius combustion method above.

DETERMINATION

Clean catalysts by boiling 10 min. in ca 6 N HNO_3 and flaming over non-luminous flame, using Pt tipped tweezers. Place catalysts in combustion tube and set tube in furnaces as shown in Figure, heat long furnace to at least 750° and preferably 800°C. or over.

Moisten entire spiral of absorber by drawing into it with gentle suction 5–10 ml of Br water for volumetric analysis or 5–10 ml. of H_2O_2 for gravimetric analysis. Care must be exercised to keep ground joint dry. Drain excess soln from absorber and attach to combustion tube with ground joint in 350° furnace. Place sample in combustion tube 5 cm from long furnace, connect O source and adjust flow to 12–15 ml/min. using flow meter or calibrated bubble counter.

Heat sample burner to at least 750°, preferably 800°C. or over, bring to 5 cm from sample and move over sample area at rate of 0.5 cm/min. (burning time 20 min.). Continue sweeping with O for 10 min. (total combustion time 30 min.). Disconnect absorber, allow joint to cool 3–5 min.

*Volumetric determination.**—Rinse contents quantitatively into 50 ml Erlenmeyer flask using 15–20 ml H_2O. Rinse outside of absorber tip. Add 5 drops Br water, boil until Br is dispelled and cool under tap. Continue as under Carius combustion method beginning with "Pour solution into titration cell, . . . "

Gravimetric determination.—Rinse contents of absorber quantitatively into previously ignited and weighed porcelain crucible using five 2 ml portions of 1:300 HCl. Place crucible on steam bath and heat to near b.p. (If total vol. exceeds 10–11 ml evap. to this vol.) Continue as under Carius Method beginning with "Add 1 ml 10% $BaCl_2$. . . ."

Alternate gravimetric determination.—Rinse contents of absorber quantitatively into 50 ml beaker with 1:300 HCl using 4–6 rinses of 3–5 ml each. Rinse outside of absorber tip. Continue as under Carius Method beginning with "heat to near 90°C. . . ."

RESULTS

The tabulated results reported for both samples by the various procedures are shown in Table 2 in which n is the number of values reported by the collaborator; $\bar{x}$, the mean of his values; s, the standard deviation of his values; $n_{\bar{x}}$, the number of $\bar{x}$ values used; $\bar{\bar{x}}$, the mean of the $\bar{x}$'s; $s_{\bar{x}}$ the standard deviation of the means; and $\bar{x}_s$, the mean of the standard deviations.

Twenty-one collaborators reported 22 sets of data for sample 1 (benzylisothiourea hydrochloride) and twenty-three sets of data for sample 2 (sulfanilamide). Values reported by collaborator 66 could not be used in the statistical calculations because they were obtained by a method similar to that described by Stragard and Safford rather than by the procedure being tested. They were included in the table for comparison, however. The 21 and 22 sets of data used in the calculations represented 97 and 109 determinations, respectively, for samples 1 and 2. The grand

* Volumetric detn. cannot be used if sample contains phosphorus.

TABLE 2.—*Data and conditions for sulfur determinations*

COLLAB. NO.	n	$\bar{x}$	s	$\bar{x}$-THEORY	CARIUS	CATA-LYTIC	TITRI-METRIC	GRAVI-METRIC	ALT. GRAV.
	Sample 1. Benzyl-iso-thiourea hydrochloride (15.82% S)								
0	7	15.80	0.08	-.02	x		x		
1	4	15.80	0.10	-.02		x	x		
2	3	15.65	0.03	-.17		x	x		
9	6	15.87	0.10	+.05		x	x		
15	4	15.73	0.05	-.09	x		x		
17	8	15.82	0.06	.00	x		x		
29	3	15.41	0.19	-.41	x			x	
30	8	15.86	0.12	+.04		x	x		
37	4	16.14	0.28	+.32	x			x	
40	4	15.77	0.12	-.05		x		x	
45	6	15.54	0.23	-.28		x	x		
46	4	15.92	0.13	+.10	x		x		
49	8	15.71	0.09	-.11	x		x		
50	4	15.83	0.04	+.01	x		x		
51	2	15.60	0.17	-.22		x	x		
59	4	15.73	0.22	-.09	x				
60	4	15.84	0.05	+.02		x	x		
60′	4	15.92	0.10	+.10	x				x
65	4	16.09	0.05	+.27		x			x
66*	(4)	(15.89)	(0.20)	(+.07)		(x)		(x)	
69	4	15.89	0.15	+.07		x		x	
8	2	15.93	0.09	+.11	x			x	
$n_{\bar{x}}$	97	21	21		11	10	13	5	2
$\bar{x}$		15.81		-0.01	15.81	15.79	15.77	15.82	16.01
$s_{\bar{x}}$		0.17			0.17	0.16	0.11	03.1	0.12
$\bar{x}_s$			0.12		0.12	0.11	0.10	0.17	0.08
	Sample 2. Sulfanilamide (18.62% S)								
0	7	18.54	0.16	-.08	x		x		
1	4	18.61	0.07	-.01		x	x		
2	3	18.66	0.04	+.04		x	x		
9	6	18.70	0.10	+.08		x	x		
15	4	18.62	0.04	.00	x		x		
15′	6	18.64	0.09	+.02		x			x
17	8	18.61	0.04	-.01	x		x		
29	3	18.31	0.06	-.31	x			x	
30	7	18.63	0.11	+.01		x	x		
37	4	18.78	0.17	+.16	x			x	
40	6	18.65	0.11	+.03		x		x	
45	6	18.48	0.06	-.14		x	x		
46	5	18.77	0.09	+.15	x		x		
49	8	18.50	0.12	-.12	x		x		
50	8	18.65	0.07	+.03	x		x		
51	2	18.72	0.04	+.10		x	x		
59	4	18.60	0.15	-.02	x				
60	4	18.66	0.09	+.04		x	x		
60′	4	18.52	0.19	-.10	x				x
65	4	18.71	0.18	+.09		x			x
66*	(4)	(18.33)	(0.30)	(-.29)		(x)		(x)	
69	4	18.80	0.19	+.18		x		x	
8	2	18.59	0.01	-.03	x			x	
$n_{\bar{x}}$	109	22	22		11	11	13	5	3
$\bar{x}$		18.62		00	18.59	18.66	18.63	18.63	18.62
$s_{\bar{x}}$		0.11			0.13	0.08	0.08	0.20	0.10
$\bar{x}_s$			0.10		0.10	0.11	0.08	0.11	0.15

* Data obtained by Stragand-Safford method and not used in statistical calculations.

mean, $\bar{\bar{x}}$, for sample 1 was 15.81 per cent sulfur as compared with a theoretical value of 15.82 per cent. The standard deviation of the means, $s_{\bar{x}}$, was 0.17 per cent, whereas the average of the s values was 0.12 per cent, indicating that the precision within laboratories was better than that between laboratories for sample 1. The $\bar{\bar{x}}$ for sample 2 was identical with the theoretical value, 18.62 per cent sulfur, and the $s_{\bar{x}}$ value was 0.11 per cent which agrees closely with the average s value, 0.10 per cent, indicating approximately the same precision within and between laboratories.

Since there was a choice as to methods (Carius or catalytic combustion) and as to the procedure for determining the sulfate formed in the combustion (titrimetric, gravimetric, or alternate gravimetric), the data for the two samples was subdivided and compared statistically. The F test (2, 4) was used as a measure of the relative precision of the results from two methods and Student's t test (2, 4) was used to compare the accuracies by determining whether or not the difference between $\bar{x}$ values for two methods was significant:

$$F = \frac{(s_{\bar{x}})_a^2}{(s_{\bar{x}})_b^2}$$

where $(s_{\bar{x}})_a^2$ is always the larger value.

$$t = \bar{\bar{x}}_a - \bar{\bar{x}}_b \sqrt{\frac{n_a n_b (n_a + n_b - 2)}{(n_a + n_b)[\Sigma(\bar{x} - \bar{\bar{x}})_a^2 + \Sigma(\bar{x} - \bar{\bar{x}})_b^2]}}$$

where $\bar{\bar{x}}_a$ and $\bar{\bar{x}}_b$ are the grand means for groups a and b; n_a and n_b are the number of values in the two groups; and $\Sigma(\bar{x}-\bar{\bar{x}})_{a\&b}^2$ are the sum of squares of the differences between the individual $\bar{x}$'s and $\bar{\bar{x}}$ for groups a and b.

These two tests were applied to the data for the Carius and catalytic methods for both samples and no significant differences in either accuracy or precision were indicated.

Comparison of the data obtained by the titrimetric and gravimetric procedures for determining sulfate, however, showed a highly significant difference in precision between the two methods, since the F values of 7.30 and 5.63 for samples 1 and 2 were even greater than 5.41, the critical F at the 2 per cent level. The lower precision of the gravimetric method shows the need for refinement of this procedure. This is essential because the titrimetric method is not applicable when the sample contains phosphorus. The difference between the means for these two sulfate methods was not critical for either sample. The alternate gravimetric results were not used in any comparisons because too few data were available. The agreement between the results obtained by those collaborators who used the alternate method, however, was good, but the $\bar{\bar{x}}$ value for sample 1 was 0.19 per cent higher than the theoretical value.

The overall $s_{\bar{x}}$ for sample 1 (0.17 per cent) was sufficiently greater than

that for sample 2 to give an F value of 2.44 which is greater than 2.09, the critical F at the 10 per cent level. Thus better precision was obtained with the more heat-stable sulfanilamide than with the more easily volatilized benzyl-isothiourea hydrochloride.

The results obtained in both the 1951 (1) and 1952 studies on micro methods for determining sulfur are summarized in Table 3. Statistical comparisons showed no significant differences in accuracy or precision

Table 3.—*Comparison of 1951 and 1952 data on sulfur determination*

		BENZYL-ISOTHIOUREA HYDROCHLORIDE				SULFANILAMIDE			
		n	$\bar{\bar{x}}$	$s_{\bar{x}}$	$\bar{x}_s$	n	$\bar{\bar{x}}$	$s_{\bar{x}}$	$\bar{x}_s$
Carius method	(1951)	7	15.79	0.12	0.12	7	18.57	0.21	0.19
	(1952)	11	15.81	0.17	0.12	11	18.59	0.13	0.10
Catalytic combustion method	(1951)	9	15.87	0.14	0.12	11	18.60	0.13	0.11
	(1952)	10	15.79	0.16	0.11	11	18.66	0.08	0.11

between the results obtained in 1951 and 1952. In 1951 the collaborators used methods then in use in their laboratory, whereas in 1952, they used the procedures described above.

CONCLUSIONS

1. The proposed Carius and catalytic combustion methods are equally satisfactory for determining sulfur.

2. The titrimetric determination of the sulfate formed by either combustion method is more precise than the gravimetric determination even though the accuracies are not significantly different.

3. The collaborators obtained as accurate and precise results by the above procedures as by the individual laboratory methods used in the previous study.

RECOMMENDATIONS*

The Associate Referee recommends—

(1) That both the Carius and catalytic combustion procedures be adopted as first action.

(2) That the titrimetric method for determining the sulfate formed by the two combustion procedures be adopted, first action.

(3) That further work be done to improve the precision of the gravimetric method which is required for samples containing phosphorus.

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REPORT ON MICROANALYTICAL DETERMINATION OF NITROGEN BY THE DUMAS METHOD

By C. L. Ogg (Eastern Regional Research Laboratory,* Philadelphia 18, Pennsylvania), *Associate Referee*

The one previous collaborative study of the Dumas method for determining nitrogen (3), made in 1948, provided data sufficient to identify only one critical variable in the method. When the data were examined to evaluate the effect of the temperature of the furnaces, two distinct groups of values were obtained; the one which represented combustion temperatures of less than 650°C. was significantly low, whereas the other, representing temperatures above 650°C., bracketed the theoretical value.

The amount of data in this latter group was not sufficient to evaluate the other variables. This year's study was therefore similar to the 1948 study except that the collaborators were asked to use furnace temperatures of 700°C. or above. Each analyst followed the procedure currently

* One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, United States Department of Agriculture.

in use in his laboratory and supplied detailed information about his procedure by filling in a standard form. Each was given two samples to analyze, nicotinic acid which was used in the 1948 study, and acetone-2,4-dinitrophenyl hydrazone. The acetone derivative replaced the benzylisothiourea hydrochloride used in the previous study so that the different procedures could be evaluated using a compound with *N-N* and *N-O* linkages, since the A.O.A.C. micro-Kjeldahl method has proved satisfactory for compounds with the *C-N* linkage. It was asked that all values be reported unless the analyst observed something unusual about a determination which would lead to an erroneous or nonrepresentative value.

Table 1 shows a summary of the data obtained for both sample 1, nicotinic acid, and sample 2, acetone-2,4-dinitrophenyl hydrazone, as well as the five variables which the statistical analysis indicated may affect the accuracy or precision of the method. In this table, n is the number of analyses reported; $\bar{x}$, the average of each collaborator's values; s, the standard deviation of his values; $n_{\bar{x}}$, the number of means; $\bar{\bar{x}}$, the mean of the $\bar{x}$'s; and $s_{\bar{x}}$, the standard deviation of the means.

Twenty-three collaborators reported 119 values for sample 1 with a grand mean of the $\bar{x}$'s of 11.40 per cent as compared with a theoretical value of 11.38 per cent nitrogen. The standard deviation of the means, 0.10, was lower than the average of the s values, 0.14. One hundred and sixteen values were reported for sample 2 by 22 analysts. The $\bar{\bar{x}}$ value was 23.47 per cent or 0.05 per cent lower than the theoretical value, whereas the $s_{\bar{x}}$ was 0.17 or higher than the average s value of 0.14. There was a significant difference in the precision of the means between sample 1 and 2 since the F value (4) or ratio of $(s_{\bar{x}})_2^2/(s_x)_1^2$ was 2.89 which is even greater than 2.79, the critical value at the 2 per cent level.

To obtain information as to the effect of the different variables in the procedure on the accuracy and precision of the results, the differences between the $\bar{x}$'s and the theoretical value were plotted as shown in Figure 1. If two groups of data obtained by alternate procedures appeared to differ in spread or location on the plot, F and/or t tests (4) were applied to determine whether or not the difference in precision and/or accuracy was significant. Twenty-four different plots were made for each sample to obtain an indication of the effect of the corresponding 24 variables. With this large number of variables and only 22 and 23 values to use, conclusive information about the variables would be impossible; however, such a treatment indicates possible sources of error or preferable procedures to use in devising a tentative A.O.A.C. procedure for subsequent collaborative study.

The first variable which was indicated to have an effect on the results was the rate of motion of the movable sample burner. Figure 1 shows the

TABLE 1.—*Data and conditions for Dumas nitrogen determination*

COLLABORATOR NUMBER		0	1	2	8	9	14	15	17	23	29*	31	37†	39
Sample 1—	n	4	4	3	2	6	9	6	8	8	3	4	6	8
Nicotinic acid (11.38%)	$\bar{x}$	11.32	11.44	11.42	11.32	11.38	11.49	11.42	11.42	11.41	11.36	11.49	11.35	11.46
	s	0.36	0.10	0.16	0.08	0.12	0.26	0.07	0.03	0.04	0.16	0.02	0.15	0.17
Sample 2—Acetone-2,4-	n	3	4	4	3	6	9	6	8	8	3	4	7	8
dinitrophenyl hydrazone	$\bar{x}$	23.34	23.60	23.54	23.40	23.64	23.56	23.48	23.77	23.27	23.41	23.44	23.63	23.96
(23.52% N)	s	0.15	0.10	0.31	0.00	0.20	0.23	0.08	0.05	0.06	0.11	0.14	0.10	0.20
Sample burner moved														
1 cm./min. or faster			—			x	x					x	—	—
Slower than 1 cm./min.		x	—	x	x			x	x	x	x		—	—
Long furnace temp.														
Higher than 750°C.				x	x		x					x		
750°C. or lower		x	x			x		x	x	x	x		x	x
Valve or stopcock														
Before comb. tube					x					x	x			
After comb. tube		x	x	x		x	x	x	x		x	x	x	x
Average sample weight														
6 mg. or over				x	x	x	x							
Less than 6 mg.		x	x						x	x	x	x	x	x
Initial rate CO_2 bubbles														
2/sec. or over			x		x	x			x		—	x	—	
Less than 2/sec.		x		x			x	x		x	—		—	x
COLLABORATOR NUMBER		45	46	51	60‡	63*	64	65	66	67‡	44	$n_{\bar{x}}$	$\bar{x}$	$s_{\bar{x}}$
Sample 1—	n	6	7	3	8	4	4	4	5	4	3	(119)		
Nicotinic acid (11.38%)	$\bar{x}$	11.23	11.46	11.29	11.32	11.44	11.49	11.63	11.23	11.39	11.35	23	11.40	0.10
	s	0.10	0.13	0.12	0.08	0.05	0.14	0.36	0.29	0.09	0.07		(0.14)	
Sample 2—Acetone-2,4-	n	6	4	3	8	6		4	5	4	3	(116)		
dinitrophenyl hydrazone	$\bar{x}$	23.36	23.48	23.44	23.40	23.45		23.46	23.81	23.34	23.54	22	23.47	0.17
(23.52% N)	s	0.20	0.17	0.09	0.12	0.09		0.17	0.13	0.12	0.14		(0.14)	
Sample burner moved														
1 cm./min. or faster		—	—		x		—	x	—	—	x		$F = \frac{(s_{\bar{x}})_2^2}{(s_{\bar{x}})_1^2} = 2.89$	
Slower than 1 cm./min.		—	—	x		x	—		—	—				
Long furnace temp.														
Higher than 750°C.				x	x									
750°C. or lower		x	x		x	x	x	x	x	x	x		$F_{0.01} = 2.79$	
Valve or stopcock														
Before comb. tube										x	x			
After comb. tube		x	x	x	x	x	x	x	x					
Average sample weight														
6 mg. or over			x	x	x		x	x						
Less than 6 mg.		x				x			x	x	x			
Initial rate CO_2 bubbles														
2/sec. or over			x	—	x	x	x							
Less than 2 /sec.		x		—				x	x	x	x			

* Zimmerman procedure (5) † Kirsten procedure (1) ‡ Shelberg procedure (2)

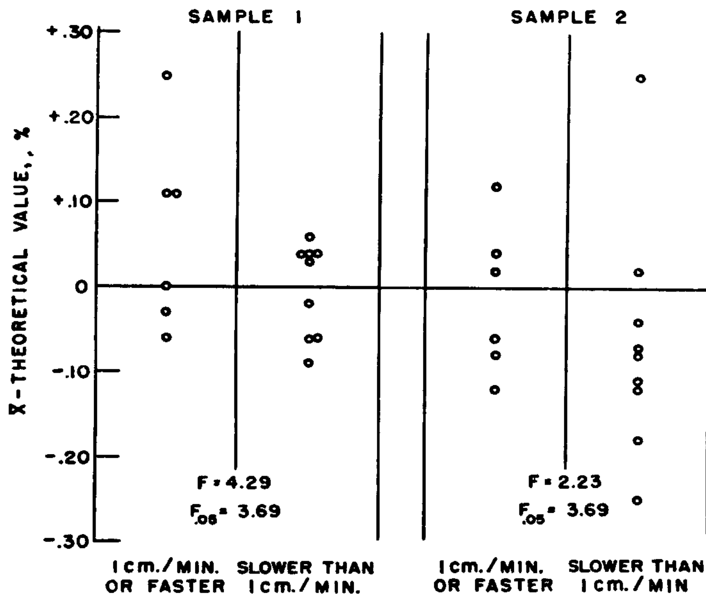

FIG. 1.—Effect of Rate of Sample Burner Movement on Dumas Nitrogen Results.

two groups of data which were obtained by those who used burner speeds of 1 cm/min. or faster and those who used speeds slower than 1 cm/min. Although the data for both samples are shown, the only significant difference was for the precision on sample 1, nicotinic acid, the more heat-stable material. Here the calculated F of 4.29 is greater than 3.69, the critical F from the 5 per cent table. Since the larger variance was arbitrarily placed in the numerator for all calculations in this paper, the critical F from the 5 per cent table indicates significance at the 10 per cent level. The data for sample 1 indicate that speeds slower than 1 cm/min. should be used, whereas for sample 2 the reverse is indicated, but in the latter case the difference in precision is not significant.

The long-furnace temperature appears to have a significant effect on precision, at least for sample 2. The data obtained from procedures using a long-furnace temperature higher than 750°C. were more precise for sample 2 than those obtained using temperatures of 750°C. and lower, as indicated by an F of 7.43 which is greater than 4.62, the critical F at the 10 per cent level. The data for both samples grouped according to this variable are shown in Figure 2. The F of 1.08 showed that the temperature effect was absent for sample 1.

Figure 3 shows the data grouped according to the location of the needle valve or stopcock used to control the flow of carbon dioxide. The F test

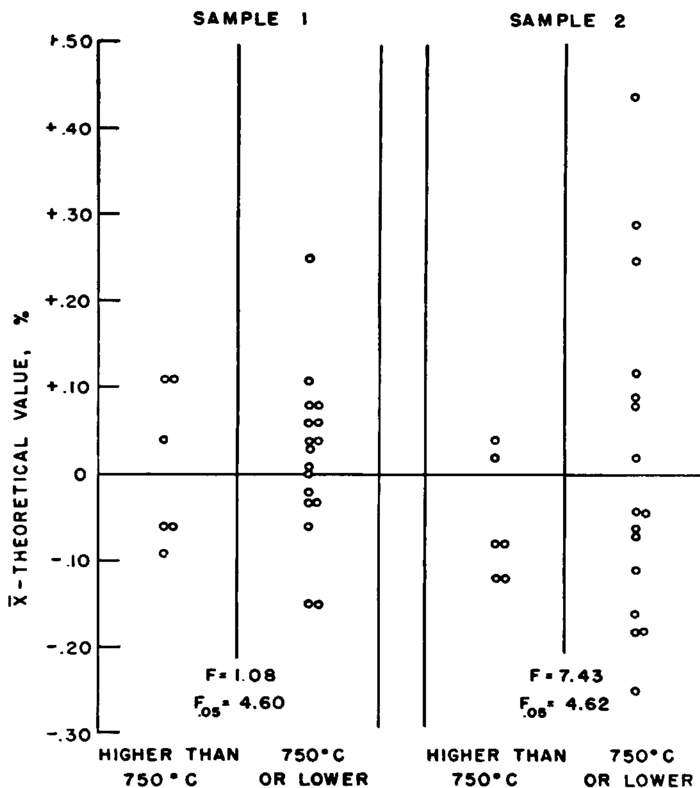

FIG. 2.—Effect of Long Furnace Temperature on Dumas Nitrogen Results.

for sample 1 shows that better precision was obtained when the controller was placed before rather than after the combustion tube. For sample 2, the F value was less than the critical F so the precision was not significantly different; however, Student's t test showed that there was a significant difference between the means for the two groups of data, with the mean for the data obtained when the controller was placed after the combustion tube being nearer the theoretical value. The possible reason for these apparent effects of the position of the needle valve or stopcock was not obvious until it was noted that half of the data obtained when the controller was placed before the combustion tube were by non-conventional Dumas procedures. Consequently the data obtained by the more conventional methods were compared with those from other procedures. This is shown in Figure 4. The "other" methods which include the Kirsten (1), Shelberg (2) and Zimmerman (5) procedures showed good precision for both samples but they were not significantly better than those obtained

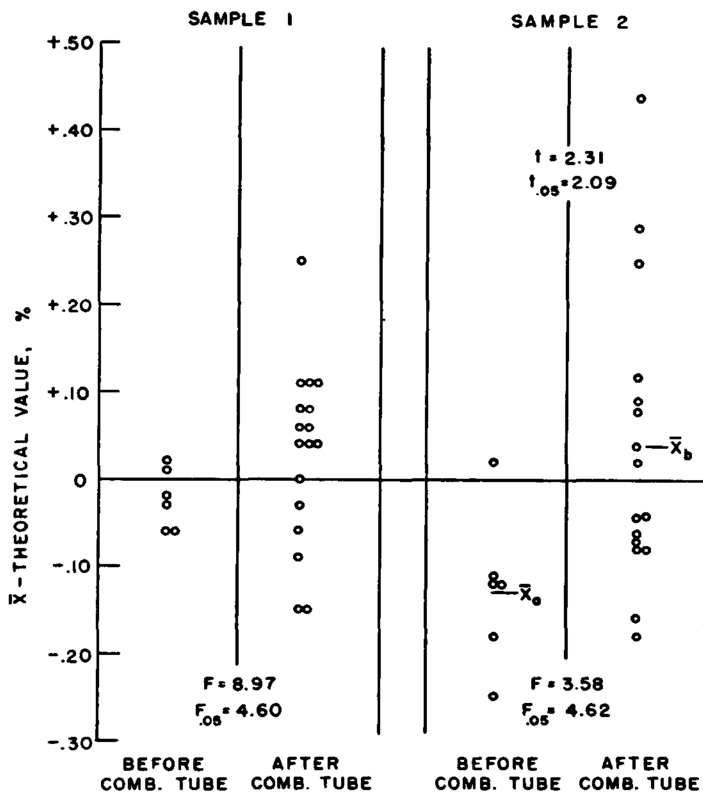

FIG. 3.—Effect of Location of Needle Valve or Stopcock on Dumas Nitrogen Results.

by the conventional methods. The pooled data for the two samples, however, did give an F of 3.35 which is greater than 2.85, the critical F at the 10 per cent level indicating better precision for the "other" methods. This leaves two alternatives, (1) to attempt to establish through collaborative study one of the newer methods (Kirsten's, Shelberg's, or Zimmerman's) as an official method, or (2) to try to improve the more usually used Dumas procedure with the aid of the results of this study. The associate referee believes that a collaborative study of any of these newer methods would not be practical until more analysts have the apparatus for and have adopted one of these procedures. Therefore, work toward generally improving the Dumas method and establishing it as an official method should continue. At the same time the referees must be alert to any trends toward more general acceptance of one of the newer methods.

Figure 5 shows the effect of "average" sample size on the precision

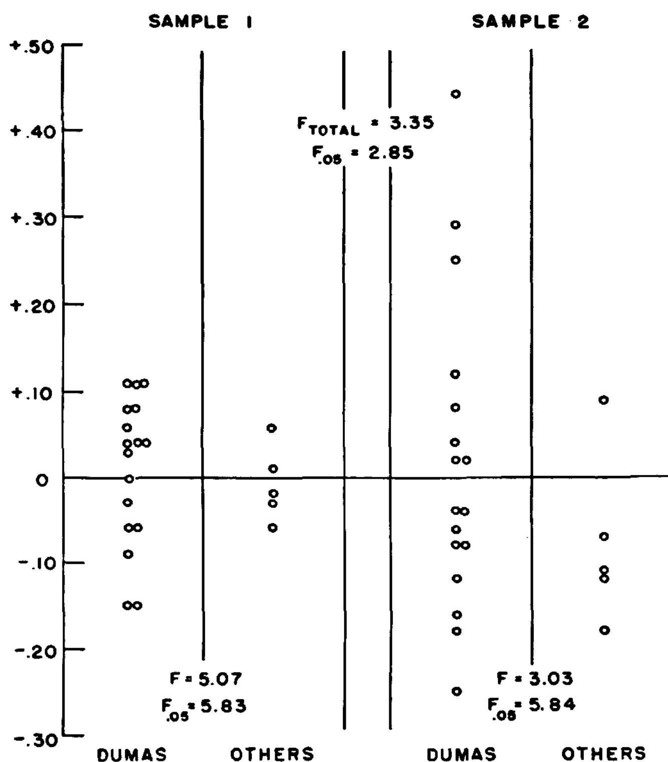

FIG. 4.—Comparison of Results from Conventional Dumas Procedures with Those From Modified Procedures.

of the results. This "average" was obtained by adding the upper and lower limits of sample weight and dividing by 2. Inspection of the plot showed that there was no effect for sample 1 but samples of 6 mg or over for sample 2 gave significantly better precision than smaller samples, because the F of 6.56 was even larger than 5.67, the critical F at the 2 per cent level.

One of the more interesting comparisons was that showing the effect of the rate of flow of the carbon dioxide used to sweep the combustion products from the tube after the sample area had been heated once. Figure 6 shows the data grouped according to flow rates of 2 bubbles per second or over and less than 2 per second. For sample 1 the F value, 3.35, was almost as large as the critical F at the 10 per cent level of 3.39, and although the F for sample 2 was considerably less than the critical value, that for the pooled data, 2.25, was again only slightly less than 2.28, the critical F at the 10 per cent level. For both samples and for the total data, flow rates of 2 bubbles per second or over tended to give more pre-

cise results. This is contrary to the older concept that the flow should not exceed three bubbles per 2 seconds and should preferably be one per second for the best results. From this study it is obvious that two or more bubbles per second can be used and that such rates may produce better results as well as reduce the time per analysis.

It must be emphasized again that the results of the statistical analysis are not necessarily conclusive because of the small number of $\bar{x}$ values as compared with the number of variables. Special attention, however, should be paid in future work to those variables apparently giving significantly better results. The other variables which were examined by plots similar to those in Figures 1 to 6 but which showed no significant differences in precision or accuracy were:

1. Gasometer versus no gasometer.
2. Dry ice versus other CO_2 sources.
3. Electric versus gas-heated sample burner.

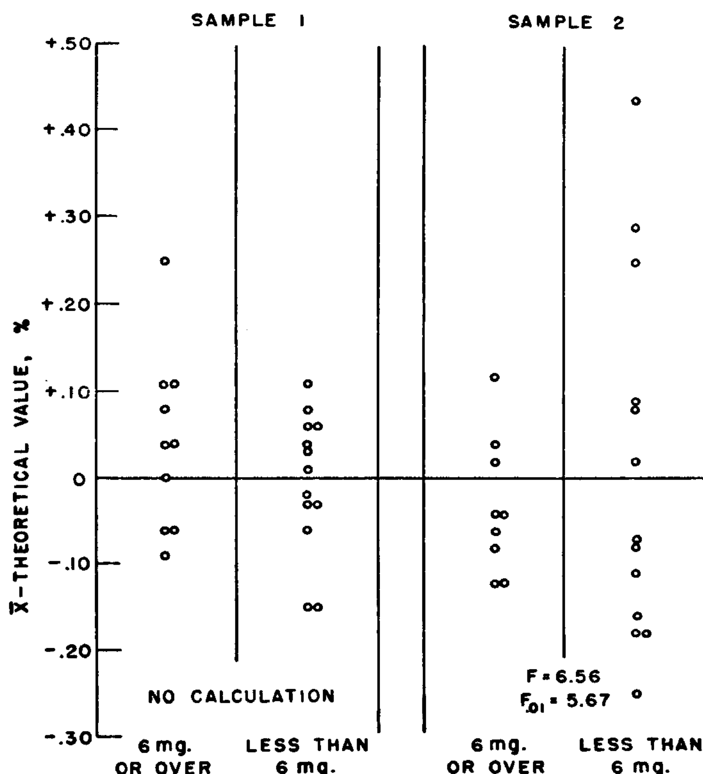

FIG. 5.—Effect of "Average" Sample Size on Dumas Nitrogen Results.

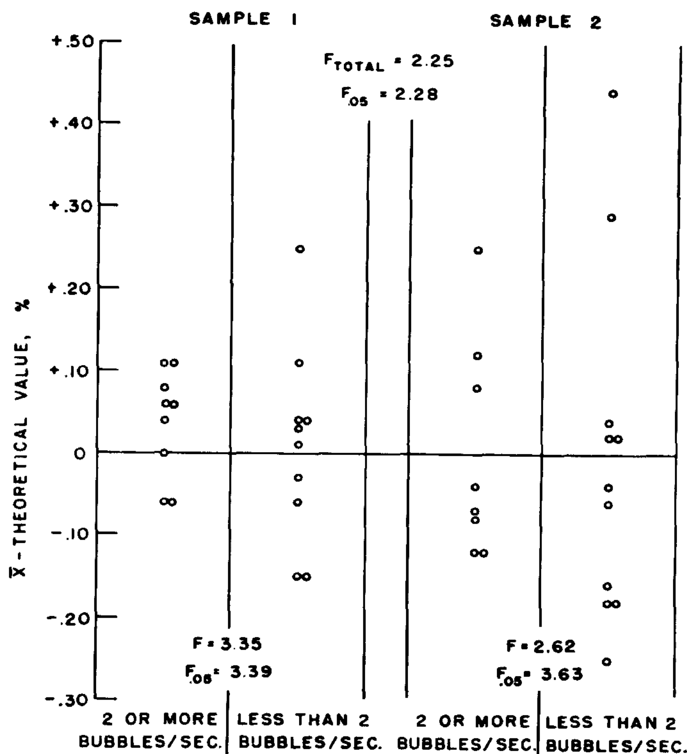

FIG. 6.—Effect of Rate of Carbon Dioxide Flow on Dumas Nitrogen Results.

4. 750°C. or higher versus lower than 750°C. sample burner temperature.
5. Electric versus gas-heated long furnace.
6. Stopcock versus needle valve.
7. Stehr (ACS) versus Pregl nitrometer.
8. Boat versus mixing tube for introducing sample.
9. Single versus double heating of sample area.
10. Glass joint versus rubber tubing to connect CO_2 controller to nitrometer.
11. 20 min. or less versus greater than 20 min. combustion time.
12. 3 bubbles or less versus greater than 3 bubbles per second final CO_2 flow rate.
13. Air temperature versus KOH solution temperature for calculations.
14. Barometer in same room versus room adjacent to apparatus.
15. True blank value versus blank calculated from control analysis.
16. Quartz versus Vycor combustion tube.
17. CuO cooled in air versus cooled in CO_2 atmosphere.
18. CuO stored in air versus stored in CO_2 atmosphere.
19. Less than 2 inch versus 2 inch or longer copper filling in tube.

The large number of variables which apparently did not cause significantly better or poorer results permits considerable freedom in devising

a tentative method for collaborative testing. It is proposed to try to strike a balance between the apparatus most commonly used and the more simple techniques with the hope that the procedure developed will prove worthy of adoption as an official method.

SUMMARY

Nicotinic acid and acetone-2,4-dinitrophenyl hydrazone samples were analyzed by 23 collaborators who reported 119 and 116 nitrogen values, respectively, by the Dumas method. Statistical analysis of the data obtained indicated that the following five variables affected the precision or the accuracy of the results: (1) Rate of sample burner movement; (2) The long-furnace temperature; (3) Location of stopcock or needle valve; (4) The "average" sample weight; and (5) Rate of sweeping with carbon dioxide.

RECOMMENDATIONS

The Associate Referee recommends* that a tentative method based on the results of this year's study be devised and subjected to collaborative test.

COLLABORATORS

J. F. Alicino, Squibb Institute for Medical Research.
V. A. Aluise, Hercules Experiment Station.
L. B. Bronk, General Electric Company.
L. E. Brown, Southern Regional Research Laboratory.
B. L. Browning, Institute of Paper Chemistry.
C. W. Clark, Picatinny Arsenal.
J. A. Corrado, Norwich Pharmaceutical Company.
Emily E. Davis, University of Illinois.
A. W. Dearing, Hunter College.
Louis Dorfman, Ciba Pharmaceutical Products, Inc.
J. R. Feldman, General Foods Corporation.
E. E. Gansel, Ansco.
G. A. Jones, E. I. du Pont de Nemours & Company.
C. W. Koch, University of California.
J. A. Kuck, American Cyanamid Company.
C. L. Ogg, Eastern Regional Research Laboratory.
J. K. Owens, E. I. du Pont de Nemours and Company.
E. D. Peters, Shell Development Company.
O. E. Sundberg, American Cyanamid Company, Calco Chemical Div.
Rita F. Preis, Smith, Kline and French Laboratories.
W. H. Throckmorton, Tennessee Eastman Company.
C. H. Van Etten, Northern Regional Research Laboratory.
I. G. Young, International Resistance Company.

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(1) KIRSTEN, W., *Anal. Chem.*, **22**, 358 (1950).
(2) SHELBERG, E. F., *ibid.*, **23**, 1492 (1951).

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* For report of Subcommittee C and action of the Association, see *This Journal*, **36**, 58 (1953).

(3) WILLITS, C. O., and OGG, C. L., *This Journal*, **32**, 561 (1949).
(4) YOUDEN, W. J., "Statistical Methods for Chemists," John Wiley & Sons, Inc., New York, N. Y., (1951), pp. 20, 25.
(5) ZIMMERMANN, W., *Mikrochemie ver. Mikrochim. Acta*, **31**, 42 (1943).

REPORT ON STANDARD SOLUTIONS

By H. G. UNDERWOOD (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Referee*

Sodium Thiosulfate Solutions.—This subject was reopened following a comment by V. A. Stenger (*Anal. Chem.*, **23**, 1543 (1951)), that thiosulfate solutions standardized by methods **39.35, 39.36** were found to give results deviating by ±0.2 per cent in analyses of a standard iodate solution by various analysts. He suggested it would be desirable to have a thorough comparison of dichromate and iodate as standards for thiosulfate under various conditions, including both the presence and absence of oxygen. Although the subject is under study, no report was received. It is recommended that the subject be continued.

Constant Boiling Hydrochloric Acid.—This subject was reopened because of an article by Liebhafsky, Pfeiffer, and Balis (*Anal. Chem.*, **23**, 1531 (1951)). Their conclusion from the data on which the AOAC method was accepted was that the normality of a single batch of constant boiling hydrochloric acid could not be guaranteed to much better than four parts per thousand. The Associate Referee modified the directions for the preparation of the constant boiling hydrochloric acid to make the method as specific as possible. The study was designed so that it would be possible to distinguish between errors inherent in the method and those of the chemists. The collaborative results support the conclusion that the error of the method is insignificant compared with the variation introduced by the standardization procedure.

The Associate Referee recommended that the proposed modification of **39.11** be adopted, first action. The referee concurs.*

REPORT ON CONSTANT BOILING HYDROCHLORIC ACID AS AN ACIDIMETRIC STANDARD

By SIDNEY WILLIAMS (Food and Drug Administration, Federal Security Agency, Boston, Mass.), *Associate Referee*, and WILLIAM WEISS (Food and Drug Administration, Federal Security Agency, Washington, D.C.)

An article by Liebhafsky, Pfeiffer, and Balis (1), which includes a statistical analysis of the work on this subject by King (2) concludes that

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 52 (1953).

the normality of a single batch of constant boiling hydrochloric acid could not be guaranteed to much better than four parts per thousand. A.O.A.C. acceptance of this method was based upon the work of King (2).

In the present work it has been the object to make the method as specific as possible and thus eliminate variations in apparatus or technique which might influence the composition of the constant boiling HCl. With this in mind, the method was rewritten to make use of an electric hotplate in place of a gas burner, and carborundum crystals in place of glass tubes. It is felt that there is less chance of superheating with the electric hotplate and that the carborundum crystals result in more even boiling than do the glass tubes of the present method.

An equation for converting observed barometric pressure to corrected pressure at 0°C. was added to eliminate the need for a table of corrections.

The revised method worked well for the Associate Referee and it was sent out to the participating laboratories along with the following directions to collaborators and notes.

DIRECTIONS TO COLLABORATORS

It is desired that two chemists at each of the participating laboratories take part in this work. Both should use the same apparatus and calibrations. In this way it may be possible to distinguish between errors inherent in the method and those of the chemist. It will also result in much more data with only a slight increase in the amount of work, since setting up and calibrating apparatus is the most time-consuming part of the work.

It is requested that:

(1) Each chemist prepare a batch of constant boiling HCl by the attached modification of **39.11** and from it prepare a standard 0.1 *N* HCl solution.

(2) Each chemist then standardize his own 0.1 *N* HCl solution and also the 0.1 *N* solution prepared by his fellow worker, in two ways:

(a) Against borax by **39.12–39.13**.

(b) Against standard 0.1 *N* NaOH (**39.10**), the NaOH having been standardized against potassium acid phthalate (**39.30–39.33**).

All titrations should be done in triplicate.

NOTES

(1) Both chemists in each laboratory should use the same equipment and the same 0.1 *N* NaOH (the NaOH being standardized by each).

(2) The hottest electric plate available (about 1000 watts) will probably give the desired distillation rate.

(3) Burettes and pipets should be calibrated. The volumetric flask used should be NBS certified. It is suggested that the volumetric flask be retained so that it can be calibrated at a later date if discrepancies are noted which might be attributed to the preparation of the solution.

(4) The barometer used should be checked against that of the local weather bureau. If the difference in height between the laboratory and weather bureau barometer is known, then a comparison can be made by phoning the

weather bureau. Otherwise, the laboratory barometer should be taken over to the weather bureau and checked with theirs.
(5) When reading the barometer, the ivory pointer in the mercury reservoir should be zeroed just before each reading.
(6) All standard solutions should be used at a temperature as close as possible to that at which they were made up to volume. Record temperature.
(7) All titrations should be made in a well-ventilated room where CO_2 will not have a noticeable effect on the titrations.
(8) Report results to 5 significant figures including the normality factors obtained for the NaOH against potassium acid phthalate.
(9) Describe in detail any unavoidable variations in procedure.

Please submit your comments and suggestions.

PROPOSED MODIFICATION OF AOAC 39.11 CONSTANT BOILING METHOD

Dil. 850 ml analytical-reagent grade HCl (35–37% HCl) with 750 ml H_2O. Check sp. gr. with spindle and adjust to 1.10. Place 1500 ml in 2-liter flat-bottom distg flask, add ca 10 carborundum crystals (ca 20 mesh) and connect to long, straight inner-tube condenser. Heat on elec. hotplate and distill at rate of 5–10 ml/min., keeping end of condenser open to air. When 1125 ml has been distd, change receivers and catch next 225 ml, which is constant boiling HCl, in Erlenmeyer flask with end of condenser inserted into flask but not below surface of liquid. Read barometer to nearest mm at beginning and end of collection of 225 ml portion and note barometer temp. Average readings.

Calc. air wt in grams (G) of this constant boiling HCl required to give one equivalent wt of HCl from the following equation (formula is applicable to pressures of 540–780 mm Hg):

$$G = \frac{P_0 + 7680}{46.839}$$

P_0 = barometric pressure in mm Hg corrected to 0°C. for expansion of Hg and of barometer scale. For brass scale barometer the following correction is sufficiently accurate:

$$P_0 = P_t(1 - 0.000162t)$$

where t = temp. of barometer in °C.

Weigh out required quantity of constant boiling HCl in tared, stoppered flask with accuracy of at least one part in 10,000. Dil. immediately and finally make to vol. with CO_2-free H_2O at desired temp.

COLLABORATIVE STUDY

Five laboratories of the Food and Drug Administration collaborated in this study. Two chemists participated at each laboratory with the exception of the Kansas City laboratory, where only one chemist was involved. Each chemist at each laboratory prepared his own constant boiling HCl. The chemist at Kansas City used pumice stones in place of carborundum crystals in preparing his constant boiling HCl. Each chemist then prepared a 0.1 *N* HCl solution and standardized it in triplicate against both borax and NaOH. Each chemist also standardized his co-worker's 0.1 *N* HCl solution in triplicate against borax and NaOH.

In all, 53 observations were made in standardizing the 0.1 *N* HCl

solutions against borax, and 55 against NaOH. The results are summarized in Table 1.

STATISTICAL ANALYSIS

The measuring stick with which the error of the method must be estimated is the standardization procedure whereby the calculated normality is checked against borax and NaOH. Since the results of the experiment are in terms of the standardized observations, any errors in this measuring stick would be reflected in an increase in the over-all variation. If the variation in normality values caused by standardization is very small, then most of the variability may be classed as the error of the method. On the other hand, if a large portion of the total variation is due to standardization, then only the remaining variation may be considered the error of the method. The problem entailed separating that portion of the variation due to standardizing the 0.1 *N* HCl solution from the error of the method of preparing the 0.1 *N* HCl. Results of the analysis are shown in Table 2.

It seems reasonable to postulate that the variation introduced by standardization may be attributable to three primary causes:

(1) The inability of the chemist to reproduce his own results exactly.

(2) The inability of a chemist to get exactly the same results as another chemist in the same laboratory.

(3) The inability of a chemist to get exactly the same results as another chemist in another laboratory.

The experiment was designed to enable us to make estimates of the first two sources of variation. Due to the limitations of time it was not considered practical to increase the work load to include the third estimate. The first estimate was available because each chemist had made several determinations from the same 0.1 *N* HCl solution, and any differences in his results would be considered due to this "within chemist" error. To obtain the second estimate, Chemist A at a particular laboratory standardized the 0.1 *N* HCl solution that Chemist B at the same laboratory had prepared. Chemist B also standardized his own 0.1 *N* HCl solution. Since both used the same 0.1 *N* HCl solution, any difference in results between the two chemists is attributable to the second source of variation. If the variation introduced by the latter is negligible, that too would be shown.

In the previous experiment (2) there was no way to separate out the second source of variation—that of the inability of a chemist to reproduce another chemist's results, because each 0.1 *N* HCl solution was standardized by only one chemist. The error introduced by standardization therefore included only the differences among determinations for each chemist, and the remainder, which included the error of the method *plus* the possible variation between chemists in standardizing, was considered to be the error of the method. This points up the necessity for designing

TABLE 1.—*Standardization of constant boiling hydrochloric acid*

LABORATORY	P	0.1 *N* HCL PREPARED BY	CALCULATED NORMALITY	NORMALITY AGAINST BORAX		NORMALITY AGAINST NaOH		NORMALITY OF NaOH AGAINST $KHC_8H_4O_4$		AVERAGE OF NORMALITIES OF 0.1 N HCl AGAINST BORAX & NaOH		
				CHEMIST		CHEMIST		CHEMIST		CHEMIST		BOTH CHEMISTS
Boston	752.2	SW	.10061	SW .10058 .10050 .10062	GPT .10058 .10064 .10064	SW .10058 .10062 .10060	GPT .10066 .10070 .10068	SW .10880 .10881 .10887 .10884 .10879 .10884	GPT .10884 .10878 .10889 .10885	SW	GPT	
		(Av.)		.10060	.10062	.10060	.10068	.10882	.10884	.10060	.10065	.10062
	752.4	GPT	.10052	.10054 .10054 .10058	.10058 .10060 .10052	.10048 .10050 .10048	.10057 .10061 .10062					
		(Av.)		.10055	.10057	.10049	.10060			.10052	.10058	.10055
Chicago	740.3	RDS	.10002	RDS .10013 .10010 .10004	AK .10005 .10006 .10003	RDS .10009 .10016 .10014	AK .09987 .09990 .09990 .10003 .10010 .10008	RDS .10023 .10018 .10020	AK .10009 .10001 .10014 .10018 .10020	RDS	AK	
		(Av.)		.10009	.10005	.10013	.09998	.10020	.10014	.10011	.10001	.10006
		AK	.10000	.10003 .10000 .10004	.10004 .10005 .10000	.09990 .09982 .10004	.09996 .09998 .10003					
		(Av.)		.10002	.10003	.09992	.00999			.09997	.10001	.09999
Denver	633.9	TJK	.09983	TJK .09993 .09992 .09988	DMT .09989 .09985 .09985	TJK .09992 .09990 .09993	DMT .09992 .09986 .09975 .09990	TKJ .10618 .10622 .10618	DMT .10617 .106285 .10611	TJK	DMT	
		(Av.)		.09991	.09986	.09992	.09986	.10619	.10619	.09991	.09986	.09989
	630.6	BMT	.10061	TJK .10062 .10060 .10060	DMT .10065 .10063 .10059	TKJ .10065 .10060 .10056	TJK .10067 .10060 .10056	TJK	DMT	TJK	DMT	
		(Av.)		.10061	.10062	.10058	.10061			.10059	.10062	.100605

TABLE 1—(*continued*)

LABORATORY	P_0	0.1 *N* HCL PREPARED BY	CALCULATED NORMALITY	NORMALITY AGAINST BORAX		NORMALITY AGAINST NaOH		NORMALITY OF NaOH AGAINST $KHC_8H_4O_4$		AVERAGE OF NORMALITIES OF 0.1 N HCl AGAINST BORAX & NaOH		
				CHEMIST		CHEMIST		CHEMIST		CHEMIST		BOTH CHEMISTS
				TS		TS		TS		TS		
Kansas City	743.3	TS	.10000	.10006		.10005		.10035				
				.10009		.10005		.10030				
				.10018		.10005		.10034				
		(Av.)		.10011		.10005		.10033		.10008		
				AL	AKK	AL[a]	AKK[a]	AL[a]	AKK[a]	AL	AKK	
Washington	763.2	AL	.10026	.10027	.10032	.10010	.10023	.10312	.10313			
				.10022	.10061	.10016	.10029	.10313	.10308			
				.10009	.10018	.10018	.10032	.10297	.10297			
					.10026							
					.10031							
		(Av.)		.10019	.10037	.10015	.10028	.10307	.10306	.10017	.10033	.10025
	762.4	AKK	.10000	.10011	.10006	.10014[b]	.10000[b]	.10537[b]	.10559[b]			
				.10004	.10002	.10008	.09994	.10556	.10565			
				.10010	.10012	.10006	.09992	.10546	.10564			
		(Av.)		.10008	.10007	.10009	.09995	.10546	.10563	.10009	.10001	,10005

(a) and (b) represent different preparations of NaOH solution.

an experiment for separating out the different sources of variation in which one is interested.

On the basis of the 0.1 *N* HCl solutions standardized by both borax and NaOH, we conclude that the error of the method is insignificant compared to the variation introduced in the standardization procedure. Standardizing against borax introduces an error, for an average of 3 determinations, of 1.4 parts per 1000, and standardizing against NaOH introduces an error of 2.8 parts per 1000. If a chemist made only a single determination, the error would be 2.1, and 3.1 parts per 1000 for borax and NaOH respectively.

The conclusions we have reached as a result of this collaborative experiment differ from those that Liebhafsky, *et al.* (1), reached in their analysis of King's data on constant boiling HCl (2). Two basic differences in the two experiments should be brought out in attempting to explain the lack of agreement.

(1) The over-all variation is smaller in the second experiment than in the first.

(2) In the second experiment, the standardization error includes a greater proportion of the total variation than has previously been estimated. Since we cannot separate out the complete standardization error in the first experiment, we can only speculate on the state of affairs in effect at that time. The reduction in the over-all variation may be due to several changes that were made in the method. These changes may have reduced the error of the method, or the error due to standardization, or both.

Since the average difference between the calculated 0.1 *N* HCl values and those arrived at by standardization against NaOH was zero, in parts per 1000, it indicated that standardizing the 0.1 *N* HCl solutions against NaOH gave an unbiased picture. On the other hand, when the solutions were standardized against borax, each laboratory gave a positive average difference of standardized normality minus calculated normality. For all of the data the average difference was 0.4 parts per 1000. On the basis of the data examined, we must conclude that this is a real difference, and that standardizing the 0.1 *N* HCl solutions against borax introduces a positive error.

In standardizing against borax, the variation introduced is approximately equally divided between that due to the differences among chemists, and that due to the differences among replicates for one chemist.

In standardizing against NaOH, the variation introduced by a chemist repeating his determinations is of the same magnitude as that introduced when standardizing against borax. However, for the NaOH data the variation among chemists is almost three times greater than the chemist's ability to reproduce his own results. The meaning of the interaction is that within a laboratory, both chemists will have either higher or lower

values when standardizing the 0.1 *N* HCl solution each prepared than the values found when each chemist standardized the other's 0.1 *N* HCl solution.

It is interesting to note that the variation due to the inability of the chemist to reproduce his own determinations is of the same order in this experiment as in that analyzed by Liebhafsky. The variation on the basis of individual determinations averages 18 parts per 10,000 in the investigation by King, 18 parts per 10,000 in this experiment on the borax data, and 17 parts per 10,000 on the NaOH data.

Standardizing NaOH against potassium acid phthalate introduces an error of 1.3 parts per 1000.

TABLE 2.—*Analysis of variance*

Borax Data[a]

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
Among laboratories	4	211.4	52.9	
Between constant boiling determinations within a lab	4	213.8	53.5	
Between chemists standardizing the same Solution within a lab	4	267.4	66.9	
Chemist×0.1 *N* HCl solution interaction within a lab	4	285.1	71.3	
Among replicates	36	1323.2	36.8	
Total variation	52	2982.0		
Bias of borax standardization	1	681.1	681.1	18.5*

NaOH Data[a]

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	F
Among laboratories	4	355.9	89.0	2.7†
Between constant boiling determinations within a lab	4	286.1	71.5	2.2
Between chemists standardizing the same solution within a lab	4	358.8	89.7	2.7†
Chemist×0.1 *N* HCl solution interaction within a lab	4	1087.6	271.9	8.3*
Among replicates	38	1244.0	32.7	
Total variation	54	3339.0		
Bias of NaOH standardization	1	6.6	6.6	

[a] The data was coded for calculation purposes by transposing the decimal point 5 places to the right.
* .1% level of significance.
† 5% level of significance.

Variance Components.—Variance components for the average of three determinations $=\sigma_0^2/3$

$$\text{Borax} = \frac{36.76}{3} = 12.25$$

$$\text{NaOH} = \frac{32.74}{3} = 10.91.$$

Variance component for the analyst interaction $= \sigma_I^2$

$$\text{Borax} = \frac{71.3 - 36.8}{3.11} = 11.09$$

$$\text{NaOH} = \frac{271.9 - 32.7}{3.23} = 74.06$$

The variation introduced in standardizing the 0.1 *N* HCl solutions:

$$\text{Borax} = 3\sqrt{\sigma_I^2 + \frac{\sigma_0^2}{3}} = 3\sqrt{11.09 + 12.25} = 14.4 \text{ or } 1.4 \text{ parts per } 1000$$

$$\text{NaOH} = 3\sqrt{\sigma_I^2 + \frac{\sigma_0^2}{3}} = 3\sqrt{74.06 + 10.91} = 27.6 \text{ or } 2.8 \text{ parts per } 1000$$

RECOMMENDATION

It is recommended* that the proposed modification of **39.11** be adopted, first action.

ACKNOWLEDGMENT

The authors wish to express their thanks to the collaborating chemists.

No report was received on sodium thiosulfate.

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REPORT ON DISINFECTANTS

By L. S. Stuart (Insecticide Division, Livestock Branch, Production and Marketing Administration, U. S. Department of Agriculture, Washington 25, D. C.), *Referee*

The results of the collaborative studies made during the year on "use-dilution" methods for evaluating disinfectants were reported, along with a detailed description of the procedure studied, in the contributed paper "Use-Dilution Confirmation Tests For Results Secured by Phenol Coefficient Methods."† As pointed out in that paper, these results indicate that the procedures described have a sufficient degree of precision to warrant acceptance for referee work. It has also been established that

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* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 52 (1953).
† See p. 464.

the results have direct application in the establishment of the dilutions necessary to secure disinfection in some commonly encountered circumstances. It is recommended†, therefore, that these methods be adopted as official, first action, for use in confirming phenol coefficient values and in determining the adequacy of solutions recommended to disinfect surfaces and articles for which prior cleaning cannot be depended upon to assure freedom from excessive organic matter or to assure a relatively low degree of bacterial contamination.

It is also recommended that the work being conducted by the Subcommittee on media ingredients for use in disinfectant testing be continued, and that the Subcommittee which has been studying the details of the official method on Fungicides extend this work to secure, if possible, collaborative data which may be necessary to substantiate such changes as may be necessary to make it a more precise procedure.

REPORT ON FUNGICIDES AND SUBCULTURE MEDIA FOR DISINFECTANT TESTING

By L. F. Ortenzio (Insecticide Division, Livestock Branch, Production and Marketing Administration, U. S. Department of Agriculture, Washington, D. C.), *Associate Referee*

During the year, studies were conducted to determine the comparative efficiency of the official spot plate method for the propagation of test cultures of *T. interdigitale* and pour plate procedures in the production of spores of uniform resistance. In the spot plate technique, the total spore count in the filtered physiological saline solution prepared in harvesting the spore crops for subsequent dilution varied from 66 million to 125 million per ml. Resistance to phenol dilutions in the standardized spore suspensions varied from 1–60 to 1–70. Greatest resistance in the standardized spore suspensions was found with spores from plates where the spore crop was the highest.

In the pour plate method, the neopeptone agar was seeded with aliquots of the standard spore suspension of 5 million spores per ml. These ranged in volume from .05 ml to 0.5 ml. The number of spores produced increased as the size of the inoculum decreased, and the resistance in the subsequently standardized suspensions was greatest when the spore crop was the highest. When a 0.05 ml inoculum was used to seed each plate, the spore crop was uniformly high. The average count of the filtered saline suspension after harvesting was 95 million spores per ml with a standard deviation of only ±8 million. The resistance to phenol was in most instances at a dilution of 1:60 but, in a few instances, a dilution of 1:65 killed the test organism.

† For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 50 (1953).

Studies should be continued on the propagation of the test culture by the pour plate method by using smaller numbers of spores for seeding the neopeptone agar plates. The objective is the development of a procedure which will give more uniform crops of spores, and spores which will have greater consistency in their resistance to phenol.

Collaborative studies were initiated on the efficiency of Congo Red as a neutralizer for use in subculture media employed in testing quaternary ammonium germicides. Results submitted by 3 collaborators showed that whereas specific batches of this dye might give excellent results in this use, other batches were ineffective in neutralizing the bacteriostatic effect of quaternaries, and some batches were actually toxic to the test culture. The only commercial preparation of this dye that gave satisfactory results was one batch of dye identified as "Merck's Reagent Grade." The great variation found in commercial lots of Congo Red would seem to rule against its use in subculture media in an official method.

The following individuals acted as collaborators in the studies outlined above:

Dr. George R. Goetchius, Rohm & Haas Company.
Dr. John F. Gain, Winthrop-Stearns, Inc.
Dr. Samuel Molinas, Food & Drug Administration, Federal Security Agency.

REPORT ON INGREDIENTS FOR DISINFECTANT TESTING

STANDARDIZATION OF BACTERIOLOGICAL CULTURE MEDIA

By MICHAEL J. PELCZAR, JR. (Department of Bacteriology, University of Maryland, College Park, Maryland), *Associate Referee*

Studies are in progress in an attempt to improve upon the medium currently used in the A.O.A.C. procedure for determination of phenol coefficients. The principal objective is to provide a medium whose constituents will lend themselves to definite characterization, and consequently to better uniformity and reproducibility.

It appeared desirable to omit the meat extract from experimental formulas since this product cannot at present be sufficiently characterized.

Initial trials were made using a single peptone (pancreatic digest of casein, U.S.P. XIII) with and without NaCl in the preparation of the test culture broth. This ingredient was selected because it represents one of the few culture media ingredients with established specifications. *Salmonella typhosa* could be maintained satisfactorily in this medium at the desired resistance to phenol (1:90) but *Micrococcus pyogenes* var. *aureus* in the same medium showed a slight loss in resistance to phenol, and varying the concentration of this peptone did not increase the resistance.

A combination of peptones has also been investigated, namely, the pancreatic digest of casein and a peptic digest of a specific animal tissue commercially available for bacteriological use. Several media have been prepared in which the ratio of the two peptones has been altered. Results have shown that the phenol resistance of both test organisms can be significantly altered as the concentration of each constituent is varied. The animal tissue peptone appeared to increase the resistance of both organisms. The exact ratio of these constituents necessary to attain the desired level of phenol resistance has not at this writing been established, but it appears possible to accomplish this in the near future.

Other peptones (where specifications can be established) singly and in combinations should also be investigated for their usefulness.

It should be emphasized, however, that no permanent improvement over the present medium can be expected unless the constituents (peptone, etc.) are characterized by acceptable specifications as to source, method of preparation, appropriate and/or distinctive chemical properties and biological performance tests.

The contributed paper entitled "Use-Dilution Confirmation Tests for Results Secured by Phenol Coefficient Methods," by L. S. Stuart, L. F. Ortenzio, and J. F. Friedl, appears on page 464 of *This Journal.*

The contributed paper, "The Resistance of Bacterial Spores to Constant Boiling Hydrochloric Acid," by L. F. Ortenzio, L. S. Stuart, and J. F. Friedl appears on page 478 of *This Journal.*

REPORT ON ECONOMIC POISONS

By J. J. T. Graham (Insecticide Division, Livestock Branch, Production and Marketing Administration, U. S. Dept. of Agriculture, Washington, D. C.), *Referee*

Early in 1952 F. I. Edwards found it necessary to resign as Associate Referee on parathion, and P. A. Giang was appointed to continue the work. Mr. Edwards' work as Associate Referee on parathion has been very much appreciated by the Association.

We are indebted to the Associate Referees for their interest and the work that they have performed. This work, as many of you know, must be done in most cases along with the Referee's regular work.

This year for the first time, we were able to have methods for allethrin, aldrin, and dieldrin studied under the direction of Associate Referees.

At the 1951 meeting two modifications of the official mercury reduction method for pyrethrins were adopted, first action. One of these modifications consisted of substitution of hydrochloric acid for sulfuric acid in the procedure for pyrethrin I. This modification eliminated the precipitation

of barium sulfate and therefore the necessity of its separation by filtration. By using this modification higher results were obtained than with the unmodified method. The fact that these results were higher was thought to be due to retention of chrysanthemum acid in the barium sulfate precipitate in the unmodified procedure. The Associate Referee for pyrethrins has discussed this modification in his report and has recommended that the action taken last year on this modification be rescinded. The Referee concurs in the recommendation and suggests that this recommendation be made effective upon adoption and this action communicated to the mailing list of *Changes in Methods.*

Volatility of the ester-type compounds of 2,4-D, 2,4,5-T, and MCP hormone herbicides is receiving considerable attention, and an attempt is being made by manufacturers to designate certain formulations as "low-volatile." Herbicides designated as "low-volatile" are generally considered safer to use near crops that are susceptible to injury from more volatile ester compounds. Biological methods of testing the volatility of these products are necessary to establish whether injury or non-injury may result from their use near susceptible plants. It therefore appears desirable that a referee be appointed to make this study.

RECOMMENDATIONS*

It is recommended—

(1) That the modifications of paragraphs **5.111** and **5.114** (pyrethrins) that were adopted as official, first action, at the 1951 meeting be dropped. It is further recommended that this action take effect immediately and that the notice of this action be sent to the mailing list of *Changes in Methods.*

(2) That a study of methods for determination of pyrethrins be continued.

(3) That the study of methods for piperonyl butoxide be continued.

(4) That the revised Elmore method for determination of organic thiocyanate nitrogen in fly sprays, as published in the report of the Associate Referee for 1950, be adopted as official.

(5) That the hydrogenation and the ethylenediamine methods of analysis for technical allethrin be further investigated and that collaborative studies be initiated.

(6) That the study of methods for isopropyl N-phenylcarbamate be continued.

(7) That methods be studied for determination of physical properties of economic poisons, especially particle size and dispersibility in aqueous and dry formulations.

(8) That a collaborative study of methods for warfarin concentrates be made.

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* For the report of Subcommittee A and action of the Association, see *This Journal*, **36**, 49 (1953).

(9) That the study of methods for analysis of low percentage warfarin baits be continued, and that collaborative study be undertaken if advisable.

(10) That the study of methods for benzene hexachloride be continued.

(11) That the study of methods for rotenone be continued.

(12) That the method for determination of potassium cyanate in herbicides, adopted as first action at the 1951 meeting, be adopted as an official method.

(13) That the method for the determination of total chlorine in esters of 2,4-D and 2,4,5-T in liquid herbicides by the Parr bomb-Boric Acid procedure be adopted, first action.

(14) That method 369 (23A Revised) (*This Journal* **33**, 767 (1950)) for determination of ester type compounds of 2,4-D and 2,4,5-T in herbicides be further studied.

(15) That the application of the partition chromatographic procedure to determination of 2,4-D and 2,4,5-T in mixtures of these herbicides be studied.

(16) That the method for parathion, adopted first action last year (*This Journal*, **35**, 65 (1952)), be modified to allow use of the potentiometric end point technique described by the Associate Referee and be further studied collaboratively.

(17) That the investigation of parathion emulsifiable concentrate analysis be continued.

(18) That a referee be appointed to study volatility of the ester forms of hormone-type herbicides by biological methods.

(19) That the study of methods for aldrin and dieldrin be continued.

(20) That an Associate Referee be appointed to study methods of analysis for systemic insecticides.

REPORT ON BENZENE HEXACHLORIDE

By Irwin Hornstein (U. S. Department of Agriculture, Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, Beltsville, Maryland), *Associate Referee*

The two colorimetric methods (1, 2) discussed in last year's report were found to be suitable for BHC residue determinations. The nonspecificity of these methods for the various benzene hexachloride isomers, however, has precluded their use for gamma isomer determinations in formulations and technical grades of BHC.

Gamma isomer determinations are at present made chiefly by infrared (3), polarographic (4), chromatographic (5), and cryoscopic (6) procedures. The infrared method meets the requirements of precision and ac-

curacy, but is too costly for most laboratories. When used alone the cryoscopic and polarographic methods are not suitable for determining the gamma content of complex formulations. The partition chromatographic procedure is now most widely used for gamma isomer determinations.

In April 1952, at a joint meeting of technical representatives of industry and government, there was informal discussion of the analytical procedures now being used for gamma isomer determinations. The chromatographic procedure as described in the *Official Methods of Analysis* (7) was generally considered to be of great utility, but in need of further study. The method gave fairly precise results in any one laboratory, but results from various laboratories did not show good agreement. In addition, the accuracy of the method appeared questionable. After chromatography, examination of the gamma fraction has been made by various laboratories using several procedures. The results are summarized below.

(a) Infrared examination showed the presence of at least four materials in the gamma fraction.

(b) Cryoscopic measurement showed melting points of about 109°C. instead of the anticipated 112–113°C.

(c) Polarographic studies indicated that the purity of the major fraction was about 90 per cent.

Other difficulties encountered were nonuniformity of silicic acid, presence of polymerizable material in the nitromethane, and difficulty in removing the chromatographed material from the column.

It was generally agreed that the chromatographic separation in combination with the cryoscopic, polarographic, or infrared method might yield reasonably accurate results. J. Rosin of the Montrose Chemical Company in an unpublished report described a method based on a chromatographic separation of the isomers and a cryoscopic determination of the purity of the main gamma fraction. The small amounts of gamma in the forerun and tailrun were determined polarographically. A committee was appointed to study the possibility of modifying the chromatographic method by some approach such as the one just mentioned. This committee is at present working in conjunction with the A.O.A.C. on a suitable procedure that will be submitted to a collaborative study.

Indications are that in the chromatographic method the main gamma fraction is about 90–100 per cent pure. In samples that contain overchlorinated materials such as heptachlorocyclohexanes, some of these are included in the main gamma fraction. However, since some small amount of the gamma isomer may appear in the forerun and tailrun, through a fortuitous combination of errors the final results may be nearly correct.

A method that was described by J. T. Craig, of the Commercial Solvents Corporation, at the same conference is also under study and may prove to be extremely valuable. This procedure is based on the principle of isotope dilution. A pure gamma benzene hexachloride containing radioactive

Cl^{36} is added to the gamma benzene hexachloride already present in the sample being analyzed. By determining the decrease in radioactivity from the standard level to the diluted level on a pure gamma fraction recovered from the mixtures, it is possible to calculate the amount of gamma benzene hexachloride in the unknown sample. This method is inherently an absolute method of analysis; the only requirement is that a weighable sample of pure gamma isomer be isolated from the mixture of standard plus unknown. The separation of this pure gamma material need not be quantitative.

At the present time methods for lindane analysis are still under study, but no recommendation can be made concerning them.

REFERENCES

(1) Phillips, W. F., *Anal. Chem.*, **24**, 1976 (1952).
(2) Schechter, M. S., and Hornstein, I., *ibid.*, **24**, 544 (1952).
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(4) Dragt, G., *ibid.*, **20**, 737 (1948).
(5) Aepli, O. T., Munter, P. A., and Gall, F. J., *ibid.*, **20**, 610 (1948).
(6) Bowen, C. V., and Pogorelskin, M. A., *ibid.*, **20**, 346 (1948).
(7) *Methods of Analysis*, 7th Ed., A.O.A.C., Washington, D. C., p. 84 (1950).

REPORT ON PYRETHRINS

By David Kelsey (Insecticide Division, Livestock Branch, Production and Marketing Administration, Department of Agriculture, Washington 25, D.C.), *Associate Referee*

Collaborative work on proposed modifications of the official mercury reduction method for determination of pyrethrins, conducted during 1951 (1), showed that a higher value for Pyrethrin I was obtained when hydrochloric acid was used for neutralization of the saponifying alkali instead of the sulfuric acid called for in the official method. The use of hydrochloric acid eliminates the need for filtering off the precipitate of barium sulfate which results when sulfuric acid is used. Analyses of the commercial pyrethrum concentrate used for the collaborative study showed an average of 1.44 per cent by weight of Pyrethrin I by the official method, and 1.65 per cent by weight of Pyrethrin I when the hydrochloric acid modification was employed. It was felt that this increase was due to active constituents which were retained by the barium sulfate precipitate when the official method was used.

The collaborative work also showed that the omission of the bicarbonate neutralization and chloroform extraction from the determination of Pyrethrin II, called for in the official mercury reduction method, did not appreciably alter the values of Pyrethrin II obtained. Using the official method, an average of 1.08 per cent by weight of Pyrethrin II was found, while an average of 1.11 per cent by weight of Pyrethrin II was obtained

when the bicarbonate neutralization and chloroform extraction were omitted from the procedure.

Following this collaborative study, the Association adopted, first action, certain revisions of **5.111, 5.112,** and **5.114** of *Official Methods of Analysis*. These revisions were:

1. The use of hydrochloric acid, in the determination of Pyrethrin I, for neutralizing the saponifying alkali.
2. The omission of the procedure involving use of sodium bicarbonate neutralization with subsequent extraction with chloroform in the determination of Pyrethrin II.

Following their publication, considerable opposition to the adoption of the first revision was expressed by the Chemical Analysis Committee of the Chemical Specialties Manufacturer's Association, which represents the principal manufacturers of pyrethrum insecticides.

In an effort to determine if any chemical explanation could be found for the increase in Pyrethrin I values, a sample of pure chrysanthemum acid was analyzed both by the official method for Pyrethrin I and by the modified method recommended by the Associate Referee. From samples containing a known amount of the pure acid, recoveries of 99.1 per cent were obtained using the official method and 99.6 per cent using the modified method. The precipitate of $BaSO_4$ obtained in the official method was dried over phosphorus pentoxide and extracted with petroleum ether. The petroleum ether was then extracted with 0.5 *N* sodium hydroxide. This extract gave a negative result when tested for the presence of chrysanthemum monocarboxylic acid with Deniges' reagent. It would appear, therefore, that while the modified method is known to give higher values for Pyrethrin I than the official method in the analysis of pyrethrins, the difference cannot be attributed to any adsorption of chrysanthemum acids by the barium sulfate precipitate.

No further work on this problem was done during 1952. The Associate Referee wishes to thank Dr. F. B. La Forge of the Bureau of Entomology and Plant Quarantine, who furnished the samples of pure chrysanthemum acid, and Mr. Robert L. Caswell of the Insecticide Division, Production and Marketing Administration, who performed the analyses.

RECOMMENDATIONS

It is recommended*—

(1) That the action of the Association in adopting, first action, the recommendations for modification of Paragraphs **5.111** and **5.114** of the Official Methods of Analysis, be rescinded.†

(2) That a further investigation of the official mercury reduction method for determination of pyrethrins should be made.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 49 (1953).
† *This Journal*, 36, 66 (1953).

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REPORT OF COLLABORATIVE STUDY ON ROTENONE

By R. Payfer (Plants Division, Department of Agriculture, Ottawa, Canada), *Associate Referee*

Through the courtesy and cooperation of Canadian Industries, Limited, a sample of ground cube root was sent to each collaborator. The original sample was mixed in our laboratory and each collaborator received about a pound of ground cube root, as well as enough carbon (Baker and Adamson; *p*H 6.6) to test six samples of rotenone. In a letter sent out at the same time as the sample, collaborators on rotenone were requested to make three tests according to the A.O.A.C. official method, and three tests according to the proposed method, with the supplied sample and carbon.

To test the effect of carbon *p*H on the extraction of rotenone, collaborators were also asked to repeat the series of determinations with the same carbon which they had been using in the past for the extraction of rotenone, and to note the *p*H of their carbon.

Only two reports were turned in and the following table shows the results. The collaborators were:

(1) Rodney C. Berry, Virginia Dept. of Agriculture, 1123 State Office Bldg., Richmond, Virginia.
(2) Howard A. Jones, U. S. Industrial Chemicals, Inc., Baltimore, Maryland.

Table 1.—*Recovery of rotenone*

COLLABORATORS	OFFICIAL METHOD		PROPOSED METHOD			
			SUPPLIED CARBON		OTHER CARBON	
	SUPPLIED CARBON[a]	OTHER CARBON	AS FOUND	CORRECTED	AS FOUND	CORRECTED
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
(1)	5.20	5.20	5.56	5.15	5.56	5.16
	5.18	5.13	5.45	5.06	5.56	5.16
	5.01	5.14	5.49	5.10	5.39	5.00
Average	5.13	5.16[b]	5.50	5.11	5.50	5.11
(2)	5.33	5.08	5.65	—	5.40	—
	5.28	4.90	5.88		5.24	
	5.27	5.00	5.87		5.24	
Average	5.29	4.99[c]	5.80		5.29	

[a] Baker and Adamson, *p*H 6.6.
[b] J. T. Baker, *p*H 4.5.
[c] Darco G-60, *p*H 5.3.

Collaborators have pointed out that a source of error in the proposed procedure is the failure to correct for the volume occupied by the sample and the carbon. Collaborator (1) states:

"For the determination of the correction needed, the following procedure was used: twenty grams of sample and ten grams of our carbon were placed in a 250 ml glass-stoppered, graduated cylinder and 200 ml of chloroform was added. This was shaken until thoroughly mixed; the volume was read as 218 ml. Therefore the true volume of solvent in the 250 ml. volumetric flask was only 232 ml."

Collaborator (2) states:

"We believe that the error in the Hageman procedure invalidates it as given in your memo. One way of accurately controlling the total amount of chloroform extract and the aliquot taken would be to make the chloroform extract, with sample and carbon present, to a definite final weight, and then take a weighed aliquot of the filtered extract. In general, we feel that the Hageman method of extraction is very promising and will certainly serve to reduce the time of analysis."

The suggested procedure was revised as given below. In the new procedure it is assumed that the filtration takes place without evaporation. The following results were obtained with this new procedure.

Rotenone: 5.12%, 5.26%, 4.99%. Average 5.12%. This average agrees with the corrected results obtained by collaborators.

REVISED HAGEMAN METHOD*

Place 20 g sample and 10 g carbon in a one liter capacity Waring Blendor. Add 300 ml $CHCl_3$ at room temp. (Check with a thermometer.) Stir 5 min. Place sample and solvent (blendor and cover) in a refrigerator (or any cool place) until cooled to original room temp. Filter mixt. rapidly into a 200 ml volumetric flask, using fluted paper without suction and keeping funnel covered with watch glass to avoid loss from evapn. Stopper flask and adjust temp. of filtrate to that of original $CHCl_3$. Transfer the 200 ml aliquot into a 500 ml glass stoppered Erlenmeyer flask and rinse volumetric flask with $CHCl_3$. Continue as stated in **5.108**—second line, starting from "and distil until only ca 25 ml. remains in flask," etc.

SUMMARY

The corrected results obtained by the revised Hageman Method agree with those by the Official Method. Not enough reports were sent in to come to a definite conclusion as to adoption. The collaborative work should be continued.

The *p*H seems to have influence on the extraction of rotenone and more work should be done on this subject.

The procedure suggested by Hornstein (*Anal. Chem.*, **23**, 1329 (1951)) for the determination of rotenone should be studied, and by combining the Hageman and Hornstein procedures, the required time for an analysis should be reduced to about eight hours.

* Hageman, R. H., *Anal. Chem.*, **21**, 530 (1949).

REPORT ON RODENTICIDES

DETERMINATION OF WARFARIN

By J. B. LaClair (California State Department of Agriculture, Bureau of Chemistry, Sacramento 14, Calif.), *Associate Referee*

During the past nineteen months this laboratory has analyzed over 41 samples of prepared rat and mouse baits containing warfarin, 0.025%, produced by 24 manufacturers. Procedure III,* Report on Rodenticides (*This Journal*, **35**, 372 (1952)) was used.

In most cases deficiencies in warfarin were easily attributed to poor mixing of bait materials or, as in several cases, deficient warfarin concentrates. Two notable exceptions to the otherwise successful analysis of all types of prepared baits submitted for analysis to date were a cracked corn bait, which we were informed had been prepared by spraying the corn with an alcoholic solution of warfarin and then a sugar syrup followed by heat drying, and a pelleted bait which had been heat dried following wet extrusion. Procedure III showed these baits to be deficient in warfarin.

As a further check of the warfarin content of these baits, bioassay tests were conducted using white rats.† These tests showed the baits to be deficient in warfarin, but that they contained approximately three times more warfarin than was found by Procedure III. During this same period the Eble method‡ for warfarin in prepared baits was announced by the Wisconsin Alumni Research Foundation. The Eble method showed the bait material to be up to guarantee in warfarin. This method is as follows:

DETERMINATION OF WARFARIN IN FINISHED BAITS

Weigh 2 g sample of bait material into a glass-stoppered bottle and add 50 ml 0.005 N NaOH or 1% $Na_4P_2O_7$. Shake one hr on a mechanical shaker. Transfer 30–35 ml to a glass-stoppered centrifuge tube and centrifuge for at least 5 min. Pipet 25 ml into a second centrifuge tube, add 5 ml 2.5 N HCl, followed by 50 ml of ethyl ether-Skellysolve B mixture (20–80), and shake 5 min. If an emulsion forms, centrifuge for a few min. Pipet 20 ml of the ether-Skellysolve layer into another centrifuge tube and add 10 ml 0.005 N NaOH or 1% $Na_4P_2O_7$. Shake for 2 min., remove the ether-Skellysolve layer, and centrifuge for a few min. with the stopper removed. Transfer an aliquot to a 1 cm cell and read in a Beckman Model DU spectrophotometer at 308 mμ against 0.005 N NaOH or 1% $Na_4P_2O_7$ (whichever was used in the detn).

Calculation: (Density − Blank§/0.459) ×0.025 = per cent warfarin.

The results of analysis on a sample of the cracked corn bait (offered for sale early in 1951) are given in Table 1.

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* This procedure has since been modified to use 10 grams of sample instead of 6 grams. The factor for converting optical density to per cent warfarin is now 0.054 instead of 0.090.

† The technique is described by Lorin R. Gillogly in "Studies of Warfarin Rat Poison," California State Department of Agriculture, *The Bulletin*, July–August–September 1952.

‡ Developed by J. Eble in the laboratory of Prof. K. P. Link, University of Wisconsin, Madison.

§ Whenever possible the same bait material without warfarin should be evaluated for most accurate warfarin value.

TABLE 1.—*Analyses of cracked corn warfarin bait* (1951)

ANALYSIS NUMBER	SAMPLE FORM AND TREATMENT	METHOD OF ANALYSIS	WARFARIN FOUND
			per cent
1	Unground sample	Procedure III	0.003
2	Ground sample	Procedure III	0.004
3	Ground sample, Soxhlet extracted with anhy. ether 28 hrs.	Procedure III	0.005
4	Ground sample	Eble method	0.021, 0.023
5	Ground sample	Bioassay	0.015
6	Ground sample	Procedure III, using ether saturated with water as extractant	0.013
7	Unground sample	Procedure III, using ether saturated with water as extractant	0.012

The results obtained in analyses 6 and 7, Table 1, would tend to demonstrate that it is necessary to remove the syrup coating before the warfarin can be dissolved by the ether.

In 1952, a new cracked corn warfarin bait was offered for sale by the same firm. This bait was analyzed and a comparison of results is listed in Table 2.

TABLE 2.—*Analyses of cracked corn prepared warfarin bait* (1952)

ANALYSIS NUMBER	SAMPLE FORM	METHOD OF ANALYSIS	WARFARIN FOUND
			per cent
1	Ground sample	Procedure III	0.014
2	Unground sample	Procedure III, using ether saturated with water	0.023
3	Unground sample	Eble method	0.025

The controversial pelleted sample was also analyzed in various ways in an attempt to determine its true warfarin content. The results are given in Table 3.

TABLE 3.—*Pelleted prepared warfarin bait*

ANALYSIS NUMBER	SAMPLE FORM AND TREATMENT	METHOD OF ANALYSIS	WARFARIN FOUND
			per cent
1	Ground sample	Procedure III	0.011, 0.012
2	Ground sample extracted 24 hrs. with ether in Soxhlet	Procedure III	0.017, 0.018
3	Ground sample	Bioassay	0.019
4	Ground sample	Eble method	0.031

The analyses of these baits presents quite a problem. There is every reason to believe that the manufacturer has used the required amount of warfarin in his product in order to meet the guarantee, and the analytical methods have yet to be proved accurate and infallible. For this reason the bioassay method was used as a referee method. This method lacks absolute precision and takes approximately a month for an analysis but it has proved most useful in evaluating both baits and methods.

Except when the Eble method was used, analyses of the early cracked and pelleted bait showed a definite deficiency in warfarin. These baits had one thing in common; both had been heated during the final stage of manufacture.

Warfarin concentrates in corn starch showed no loss of warfarin when heated as long as three hours at 100°C., yet when moistened with water or alcohol to a paste and heated, they lost as much as 40 per cent of warfarin.

Due to absorbance of bait components other than warfarin the question of the proper blank value to be subtracted from the optical density reading at 308 mμ presents a serious problem. Procedure III and the two ether extractions of the sodium pyrophosphate solution of warfarin removes most of the interfering substances from most baits; yet there are many commercial baits containing everything from dyes to rancid lard. Many of these materials are of an acidic nature and follow along with the warfarin to give high values.

Instead of trying to calculate a blank value from the components of a bait, it was decided to try to eliminate interference by adsorbents.

Two grams of the adsorbent were suspended in ethyl ether and transferred to a chromographic tube 11 mm I.D.×200 mm. long, fitted with a capillary stop-cock. (A plug of glass wool holds the adsorbent in place.) Using air pressure, the excess ether was forced through the tube until the adsorbent bed was just covered. A two ml aliquot of a warfarin solution in ether was carefully added to the tube so as not to disturb the bed, the ether solution was forced into the bed, and the tube filled with ether. Air pressure was applied to the top of the column so that the ether passed out of the column at about two drops per second. Cuts were taken every two milliliters in glass-stoppered mixing cylinders. After each cut was made, 5 ml. of 1% sodium pyrophosphate solution was added to the cylinder, and after shaking two minutes and centrifuging, the ether was drawn off. The aqueous solution was then extracted with 2 ml purified petroleum ether, centrifuged, and the petroleum ether removed by aspiration. Each cut was then placed in the Beckman Model DU spectrophotometer and the optical density at 308 mμ was determined. The results obtained on the two most promising adsorbents are given in Tables 4 and 5.

To test the efficiency of the adsorbents in removing interference from warfarin solutions, a 0.025 per cent warfarin bait was prepared with sardine meal, which is known to cause severe interference.

A 10 g weight of the fish meal-warfarin bait was shaken for one hour with 50 ml ethyl ether and a 2 ml aliquot of the ether solution was passed through the chromatographic column. In the case of the silicic acid, 17 ml of ether was passed through,

TABLE 4.—*Passage of warfarin in ether solution through silicic acid**
(2 ml. warfarin solution containing 0.0832 mg. warfarin per ml. in ether)

CUT NUMBER	VOL. OF CUT	WARFARIN IN CUT	RECOVERY
	ml	*mg*	*per cent*
1	2.00	0.0037	2.22
2	2.00	0.0524	31.47
3	2.00	0.0927	55.68
4	2.00	0.0138	8.29
5	2.00	0.0023	1.38
6	2.00	0.0012	0.72
7	2.00	0.0004	0.24
8	2.00	0.0000	0.00
Totals		0.1665	100.00

* Mallinckrodt, 100 Mesh, $SiO_2 \cdot XH_2O$, Analytical Reagent.

TABLE 5.—*Passage of warfarin in ether solution through Attapulgus clay**
(2 ml. warfarin solution containing 0.0732 mg. warfarin per ml. in ether)

CUT NUMBER	VOL. OF CUT	WARFARIN IN CUT	RECOVERY
	ml	*mg*	*per cent*
1	2.00	0.0000	0.00
2	2.00	0.0000	0.00
3	2.00	0.0010	0.68
4	2.00	0.0119	8.13
5	2.00	0.0539	36.82
6	2.00	0.0258	17.62
7	2.00	0.0139	9.49
8	2.00	0.0137	9.36
9	2.00	0.0130	8.88
10	2.00	0.0073	4.99
11	2.00	0.0035	2.39
12	2.00	0.0022	1.50
13	2.00	0.0000	0.00
14	2.00	0.0000	0.00
Total		0.1462	99.86

* Attapulgus clay, grade 200/up, Attapulgus Clay Co., 210 W. Washington Square, Philadelphia 5, Pennsylvania.

while 25 ml was passed through the Attapulgus clay column. The ether from the column was caught in 50 ml glass-stoppered mixing cylinders. After adding 10 ml of 1% sodium pyrophosphate solution and shaking two minutes, the analysis was continued as in the regular Procedure III method.

The results obtained on the fish meal bait are shown in Table 6.

SUMMARY

There has been controversy over the methods of analysis applicable to cracked corn and pelleted warfarin baits. It appears that further

TABLE 6.—*Comparison of methods of purification on the analysis of a fish meal bait containing 0.025% warfarin*

ANALYSIS NUMBER	PURIFICATION METHOD	METHOD OF ANALYSIS	WARFARIN FOUND
			per cent
1	None	Procedure III	0.054
2	None	Eble method	0.064
3	2 g silicic acid	Procedure III	0.029
4	2 g Attapulgus clay	Procedure III	0.025

development of procedures is necessary before they can be put to collaborative study.

There seems to be little doubt that the cracked corn bait made in 1952 contained more warfarin than the 1951 bait, yet the Eble method gave practically the same results on both samples.

The Eble method is more subject to interference than Procedure III, and a blank correction must be applied in almost every case.

The substitution of ether saturated with water for the dry ether as an extractant in Procedure III makes this procedure applicable to the cracked corn bait.

Ether extraction in a Soxhlet apparatus appears to extract completely the warfarin from the ground, extruded, pelleted bait.

Heat treatment of baits during manufacture seems to cause a loss of some of the warfarin. The loss depends upon time, temperature, and moisture content. The actual reason for the loss has not been studied, but it appears to be due to volatility rather than decomposition.

The use of Attapulgus clay as a means of purifying the ether extracts of warfarin baits should greatly increase the accuracy of analysis of some types of prepared baits.

The sample weight for 0.025 per cent warfarin baits should be changed to 10 grams per 50 ml of ethyl ether, instead of 6 grams as was reported in last year's rodenticide report. This concentration places the spectrophotometer readings in a more accurate range. The optical density readings are multiplied by the factor 0.054 instead of 0.090.

RECOMMENDATIONS*

It is recommended—

(1) That a collaborative study of warfarin concentrates be undertaken.

(2) That work be continued to check and improve existing methods for the analysis of low-percentage warfarin baits. If these methods appear accurate enough, a collaborative study should be undertaken.

* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 49 (1953).

REPORT ON 2,4-D HERBICIDES

By A. B. HEAGY (Maryland Inspection and Regulation Service, College Park, Md.), *Associate Referee*

In the continuation of the program, started in 1949, to devise test methods for analysis of 2,4-D herbicides and related compounds, four procedures and material were supplied to eighteen collaborators. Of this number nine completed some part of the project.

Method 369 (23-A Revised) for the determination of esters of 2,4-dichlorophenoxyacetic acid.*—Seven workers reported results of their experience with this test, on a material having a theoretical 2,4-D content of 37 per cent, as follows: 41.42, 41.41, 40.24, 39.22, 38.38, 37.60, 37.36, 36.76, 36.92, 36.42. *Average* = 38.57.

It is noted that the deviation from the average is +2.9 and −2.1. A comparable product was examined in 1951 by eight workers whose figures varied +0.5 from the average value.

Revisions based on collaborators' recommendations were made in the procedure studied this year. It is apparent that some phase of the present approach is in error, which leads us to believe that no definite suggestions can be made for it in its present form.

Method 370 for the determination of total chlorine in liquid herbicides 2,4-D, 2,4,5-T, or mixtures of both in the presence of oils and emulsifiers, by a modified Parr bomb procedure.*—Results reported by four collaborators were: 39.15, 39.00, 38.84, 38.60, 38.76, 38.27, 37.76. *Average* = 38.62.

It is observed that quite a variation exists in the figures reported. The prolonged drying period necessary to eliminate the oil carrier is the main objectionable feature of the test. However, analysts report successful use of the method to check results obtained by other means. The majority opinion is that the time-consuming drying period offsets other good features of the procedure.

Percentages this year varied slightly more from the general average than those of last season and in view of the apparent variations and stated objections no recommendations are made for this procedure.

Titration of the acid group applicable to mixtures of esters of 2,4-D and 2,4,5-T.—This procedure is a variation of the extraction method and involves adjustment of the pH during various phases of the routine. It was distributed as an alternate method and nine results were reported as follows: 39.60, 39.66, 39.55, 39.33, 39.04, 38.84, 38.70, 38.55, 38.46. *Average* = 39.08.

Three definite ranges of figures were obtained with a spread of over 1 per cent between the high and low. The test shows possibilities, but no definite recommendations seem justified at this time.

* *This Journal*, **33**, 767 (1950); **34**, 674 (1951); **35**, 377 (1952).

Determination of total chlorine in esters of 2,4-D in liquid herbicides by Parr bomb-boric acid.—This procedure was devised by Clemens Olsen of the Arizona Inspection Laboratory. He observed that many chemists were constantly searching for a material to insure complete combustion in the Parr bomb. Silica proved successful as an aid to combustion but its removal from solution was laborious and time consuming. Therefore, boric anhydride was tried and proved to be beneficial as an aid to complete combustion and was sufficiently soluble to cause no difficulty in the determination of the halogens. Boric anhydride was prepared by drying boric acid at temperatures of 120°C. to 220°C. for approximately two weeks. (Investigation revealed that Eimer & Amend manufacture Boric Anhydride fused, lump, pure B_2O_3—mol. wt. 69.94. This product proved satisfactory.)

METHOD

DETERMINATION OF TOTAL CHLORINE IN ESTERS OF 2, 4-D AND 2, 4, 5-T, BY PARR BOMB-BORIC ACID PROCEDURE

To 1.5 g of boric anhydride (Eastman Kodak Co., Cat. #2685 or equivalent) contained in a 42 ml Parr bomb, elec. ignition type, add from a small weighing burette ca 0.25–0.30 g sample contg 0.030–0.034 g Cl. (When a sample larger than 0.30 g is required, 2.5 g boric anhydride should be used. In no case should a sample larger than 0.6 g be taken.) Mix well with a thin stirring rod. Measure 15 g of calorimetric grade Na_2O_2 in a standard measuring dipper, add a small portion to contents of the bomb, and stir. Add balance of Na_2O_2 and thoroly mix by stirring with rod. Withdraw rod and brush free of adhering particles. Quickly cut or break off lower 1½″ of stirring rod and imbed infusion mixture. Prep. head by heating fuse wire momentarily in a flame and immersing it into a small quantity of sucrose. One mg sucrose is sufficient to start the combustion. Assemble bomb and ignite in usual manner.

Place ca 100 ml of distd H_2O in a 600 ml beaker and heat nearly to boiling. After cooling bomb, dismantle and dip cover in the hot H_2O to dissolve any of the fusion which may be adhering to the underside. Wash cover with fine jet of distd H_2O catching washings in the beaker. With tongs, lay fusion cup on side in the same beaker of hot H_2O, covering it immediately with watch glass. After fused material has dissolved, remove cup and rinse with hot H_2O, cool soln, add several drops of phenolphthalein indicator, neutralize with concd HNO_3 and add 5 ml in excess. Det. Cl by electrometric titration or by Volhard procedure **5.148(a)** or **(c)**.

Run a blank which includes all reagents used.

Subsequent work indicates that a mixture of 1 g of finely powdered KNO_3 and 0.4 g of finely powdered sucrose is necessary to insure complete combustion with certain products.

Of the eight results reported a variation from 40.10 to 39.25 was found: 40.10, 39.57, 39.48, 39.44, 40.06, 39.54, 39.46, 39.25.

If high figures are eliminated the average is 39.47. Three collaborators obtained results that checked within 0.2 per cent. Collaborators expressed satisfaction with the procedure because of its speed and simplicity of operation.

Determination of 2,4-D ester materials by use of an ion exchange column.—Last year in the Associate Referee's report, attention was directed to a

new approach to the 2,4-D ester group examination. This procedure was sent out for trial where time permitted. Two results were reported as follows: 41.49 and 41.35 per cent. These figures indicate that the method is prone to give high results and no recommendations are made at this time.

After the current project had begun, a copy of a paper presented by S. W. Stroud, Boots Pure Drug Co., Ltd., Nottingham, England, to the Society of Public Analysts and Other Analytical Chemists at their annual convention, November 7, 1951* was received by the Associate Referee. This report concerned the separation and determination of 2,4-D in a mixture of chlorinated phenoxyacetic acids. It is based on the separation of the acids by partition chromatography between ethyl ether and strong phosphate buffers on a "Hyflo-Supercel" column and their determination by titration of the carboxylic acid groups. It was the opinion of the author that the individual acids can be determined rapidly on 10 to 20 mg of sample. No practical advantage resulted from the use of an indicator to bring out the various bands in the separation. Complete determinations could be carried out in half an hour to an hour.

COLLABORATORS

The Associate Referee wishes to express his appreciation to the following for their collaboration in this study:

J. M. F. Leaper, American Chemical Paint Co., Ambler, Pa.
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W. R. Flach, Eastern States Farmers' Exchange, West Springfield, Mass.
H. A. Thomson, Kaugatuck Chemicals Division, Dominion Rubber Co.
Clemens Olsen, Arizona Inspection Laboratory.
Herbert A. Rooney, Bureau of Chemistry, California Department of Agriculture.
A. C. Keith, Kansas Control Division, Board of Agriculture.
Howard Hammond, State Laboratories Dept., North Dakota.
Romeo Payfer, Department of Agriculture, Ottawa 2, Ontario, Canada.
Boyd L. Samuel, Division of Chemistry, Dept. of Agr. & Immigration, Virginia.

RECOMMENDATIONS†

1. In view of the discrepancies in the figures reported for method 369 (23A Revised) no recommendations are made for this procedure. However, it should be noted that in its original form (of 1951) it has proved adequate for some types of ester materials and should be retained for spot use.

2. That the method for the determination of total chlorine in esters of 2,4-D and 2,4,5-T or mixtures of both in liquid herbicides by the Parr bomb-boric acid procedure be adopted as official, first action.

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* *Analyst*, 77, 63 (1952).
† For report of Subcommittee A and action of the Association, see *This Journal*, 36, 50 (1953).

3. That the partition chromatographic procedure be investigated to obtain a method applicable to mixtures of 2,4-D and 2,4,5-T and their quantitative estimation.

(4) That the method* for the determination of potassium thiocyanate in herbicides be adopted as official.

REPORT ON DETERMINATION OF ISOPROPYL N-PHENYLCARBAMATE AND RELATED COMPOUNDS

By Roy L. Shaw (State of Oregon, Department of Agriculture, Salem, Oregon) *Associate Referee*

The increased prominence of isopropyl N-phenylcarbamate (IPC) and related compounds as selective weed control chemicals has necessitated the development of an analytical method. The Associate Referee has reviewed and considered three possible approaches to the problem: 1. The determination of nitrogen or chlorine, in the case of chloro-compounds, with the results calculated in terms of IPC or 3-chloro-IPC; 2. The determination of aniline liberated when IPC is treated with a phosphoric acid-sulfuric acid mixture (1); 3. The determination of carbon dioxide liberated when IPC is treated as in 2 (1, 3, 4).

DISCUSSION

The determination of IPC by the first method is not entirely satisfactory. The large conversion factor used in calculating the purity of the product often makes duplication difficult. In liquid products, where various commercial solvents and emulsifying agents are present, erroneous results due to interference from these reagents are often obtained. The chlorine derivatives are very difficult to determine by a total chlorine method.

The determination by the aniline method for micro quantities in spray residue work (1) has been published. No work was done on this method in our laboratory.

Gard (2) has presented a method of assay for IPC based on the determination of CO_2. This report summarizes his method and gives variations tried by the Associate Referee.

METHOD (2)

APPARATUS

Fig. 1 shows the apparatus used by Gard.

PROCEDURE

A 2.5–3.0 g sample is placed in reaction flask C with 5 ml of distd H_2O and 4–5 glass beads. Connect flask to app. Absorption tower E is charged with 40.0 ml of

* *This Journal*, **35**, 63 (1952).

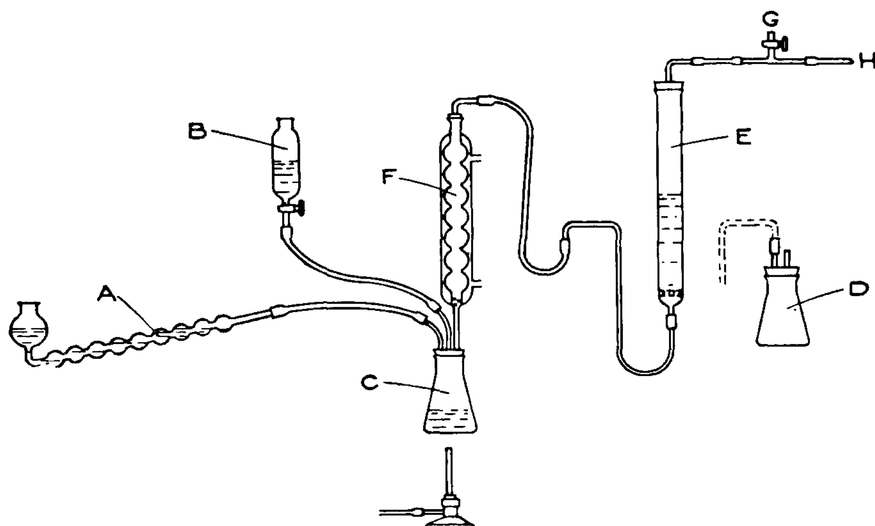

FIG. 1.—Gas Absorption Apparatus for Hydrolysis. A. Meyer sulfur bulb, B. Acid reservoir, C. Reaction flask, D. Reservoir flask, E. Absorption tower, F. Condenser, G. Stopcock, H. Aspirator connection.

standard N NaOH soln. Apply suction to system by adjusting the H_2O aspirator so that bubbles rise continuously thru absorption tower. Add 30 ml H_3PO_4-H_2SO_4 mixt. (91 ml 85% H_3PO_4+9 ml H_2SO_4) to the reaction flask. Heat gently until initial evolution of gas has ceased. Continue to boil gently for 45 min. Transfer soln from the receiver flask to a 600 ml beaker, dil. to ca 400 ml. Add 50 ml 10% $BaCl_2$ and mix vigorously for 1 min. Add 2 ml phenolphthalein indicator soln and titrate the excess NaOH with standard N HCl. Process a blank in same manner as above sithout sample. Correct titration for blank.

$$\text{Calculation: Per cent } IPC = \frac{\text{ml NaOH (net)} \times N \times 0.08961 \times 100}{\text{wt of sample}}.$$

The Associate Referee found this method to be effective for technical grade IPC. For 3-chloro IPC and various liquid formulations of IPC the results appeared to be low. The modifications the author made for the following determinations (see Tables 1–3) were as follows:

1. The substitution of 20 ml of ethyl acetate for the 5 ml of water. This serves continuously to wash down any sublimed IPC which may collect in the reflux condenser.

2. The addition of two sulfuric acid scrubber tubes placed between the reflux condenser and the absorption flask. The purpose of these is to absorb any ethyl acetate or other solvents used in the formulation of a liquid IPC product which might escape the reflux condenser and act as an interfering substance. In the author's laboratory, scrubbers were made from 8 inch side-neck test tubes. The sample weight was reduced to 1–2 g. The sodium

hydroxide was also reduced to 0.1 *N* which was found to be of sufficient strength. The above modification was also applied to technical grade IPC with favorable results.

3. As stated in the quoted paper (2), 45 minutes is an adequate time for IPC. However, it was found by the Associate Referee that this is not adequate for the 3 chloro-derivative which may require up to 1½ hours for complete decomposition.

TABLE 1.—*Determination of IPC 3 times recrystallized from ethyl alcohol*

SAMPLE NO.	TIME	GARD'S METHOD	MODIFIED METHOD
	min.	*per cent*	*per cent*
1	45	99.1	99.6
2	45	99.3	99.5
3	45	—	99.3

TABLE 2.—*Analysis of 20 per cent commercial IPC liquid formulation*

SAMPLE NO.	TIME	GARD'S METHOD	MODIFIED METHOD	NITROGEN DETN
	min.	*per cent*	*per cent*	*per cent*
1	45	19.12	20.71	20.18
2	45	19.46	20.50	21.22
3	45	19.78	21.02	21.82

TABLE 3.—*Analysis of a commercial 3-chloro IPC liquid formulation*

SAMPLE NO.	TIME	GARD'S METHOD	MODIFIED METHOD	CHLORINE DETN
	hrs.	*per cent*	*per cent*	*per cent*
1	¾	23.1	25.6	47.2
2	1½	36.1	39.1	39.8
3	1½	35.2	38.6	37.7
4	1½	—	38.4	40.7

The theoretical problems discussed by Gard are not mentioned here. The rate of aspiration was not controlled carefully in the above analyses and this may account for some variation in results.

In view of the fact that a very limited amount of study has been made of this analysis, it is recommended* that continued study be made by the Association.

ACKNOWLEDGMENT

The author wishes to express his thanks to J. D. Patterson, Chief Chemist of this laboratory, and Virgil G. Hiatt, Assistant Chief Chemist, for their help and cooperation.

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 49 (1953).

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REPORT ON PARATHION

By Paul A. Giang (U. S. Department of Agriculture, Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, Beltsville, Md.), *Associate Referee*

Last year it was recommended that the O'Keefe and Averell (1) titration method for the analysis of technical parathion and parathion formulations be adopted, first action (2). Most of the collaborators last year, however, were not satisfied with the titration end point of the method. The difficulty, according to the reports, lies in the determination of the exact shade of color which should be taken as the end point. They complained that the end point seems to vary in tests performed by different individuals and also to vary from day to day in tests made by the same individual. It was suggested, therefore, that the titration in the O'Keefe and Averell method be further studied in the hope of finding some technique which will give a more precise and more reproducible end point.

H. A. Thomson, one of the collaborators, suggested the use of a potentiometric titration method such as the one used by La Rocca and Waters (3) in their work on sulfa drugs. Both Edwards, the former Associate Referee on parathion, and the present writer have tried this potentiometric titration technique and are very satisfied with it.

Last June samples of a technical parathion and a 25 per cent wettable powder were sent to twelve laboratories for collaborative study. Seven laboratories reported their results. Four of these laboratories used the potentiometric titration technique, one tried both the potentiometric technique and the potassium iodide-starch paper technique, one used the potassium iodide-starch technique alone, and one did not specify in its report the titration method used. The results are shown in Tables 1 and 2.

In general, all the collaborators who reported results obtained with the potentiometric technique were quite satisfied with it. Their comments are that the end point was sharp and well defined and that no special difficulty was encountered. The results obtained with the potentiometric technique as reported from various laboratories are surprisingly close and uniform.

The O'Keefe and Averell titration method, which was used for this year's collaborative studies, was described in last year's report (2);

TABLE 1.—*Collaborative results utilizing potentiometric methods*[a]

ANALYST	METHOD USED	TECHNICAL PARATHION		WETTABLE POWDER	
		PARATHION	P-NITROPHENOL	PARATHION	P-NITROPHENOL
1	Max. potential rise	97.60		23.50	
		97.66		23.50	
		97.54		23.55	
	Dead-stop end point	97.55		23.50	
		97.59		23.50	
		97.64		23.45	
2	Dead-stop end point	97.50	0.053		
3	Max. potential rise	98.00	0.06	23.56	1.20
		97.89	0.07	23.79	1.16
		98.23	0.06	23.79	1.26
4	Max. potential rise	97.86	0.072	23.58	1.20
		97.54	0.069	23.54	1.18
		97.68	0.070	23.60	1.14
		97.76	0.070		
		97.70	0.064		
		97.68	0.070		
Average		97.72 ±0.05		23.57 ±0.03	

[a] Note: One collaborator, using the maximum potential rise titration technique, obtained the following results on the wettable dust sample of parathion without correcting for the free nitrophenol content:
Titration on the first day—25.1, 25.3%
Titration on the second day—25.6, 24.7%

TABLE 2.—*Collaborative results utilizing other titration methods*

ANALYST	METHOD USED	TECHNICAL PARATHION		WETTABLE POWDER	
		PARATHION	P-NITROPHENOL	PARATHION	P-NITROPHENOL
1	Starch-iodide	97.70	0.048	23.40	1.29
		97.60	0.049	23.40	1.29
		97.40			1.31
2	Starch-iodide	96.2		23.60	
		95.6		23.70	
		95.9		23.90	
3	Not given	98.14		23.24	
		98.22		23.01	
		98.11			
		97.67			
Average		97.25 ±0.31		23.46 ±0.11	

therefore, only the potentiometric tritation technique as given by La Rocca and Waters (3) is outlined here, as follows:

TITRATION TECHNIQUE

APPARATUS

Beckman potentiometer (equipped with an adaptor for outside electrodes) or some other type of potential titrimeter, a platinum electrode, a calomel electrode, and a suitable stirrer.

PROCEDURE

Place the electrodes and stirrer in the reaction mixt. which has been reduced (by the O'Keefe and Averell method) and cooled to room temp. Add 5 g of potassium bromide to the mixt., start the stirrer, and then add the standard sodium nitrite soln in 5-ml portions up to within 1 ml of the calcd equivalence point. From this point on, add the nitrite in 0.1-ml portions until a maximum rise in potential is achieved. At first the potential after each addn of the nitrite soln requires some time (3 to 5 min.) to become constant; however, as the equivalence point is approached, especially after the 0.1 ml addns, the reaction is completed within 1 min.

In this technique no attempt is made to titrate to a definite potential, but rather to titrate to a maximum rise in potential. Potassium bromide is needed in order to accelerate the reaction. At room temperature it was found that only a negligible side-reaction occurred; it is not necessary, therefore, to cool the reaction mixture in ice during the titration (4). The potentiometer readings should be recorded whenever the potential becomes a steady value; thus, the sharp rise in potential at the end of titration is easily recognized. At the end point, the addition of less than 0.1 ml of sodium nitrite solution will cause a sharp rise in potential.

After the end point is reached there will again be a slight rise in the potential after each addition of the nitrite, but with further addition the potential will assume a constant value, and a steady fall will follow if still more of the nitrite is added.

Two laboratories also reported the use of the dead-stop end point technique, as described by Scholton and Stone (5). This technique as reported is, in general, equally as good as the maximum rise in potential technique; the choice of one or other of these two techniques is entirely dependent on individual preference and the type of potentiometric apparatus available.

RECOMMENDATIONS*

(1) That the method for parathion, adopted first action last year, be modified to use the potentiometric end point technique described by the Associate Referee and be further studied collaboratively.

(2) That the investigation of the analysis of parathion emulsifiable concentrate be continued.

COLLABORATORS

The Associate Referee wishes to thank the following collaborators for their cooperation in this year's study.

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 50 (1953).

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Calvin H. Schmiege, Mathieson Chemical Corporation, Niagara Falls, New York.
Lloyd G. Keirstead, Connecticut Agricultural Experiment Station, New Haven, Connecticut.
H. A. Thomson, Naugatuck Chemicals, Elmira, Ontario, Canada.

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REPORT ON ORGANIC THIOCYANATES

By Herbert A. Rooney (California State Department of Agriculture, Bureau of Chemistry, Sacramento, Calif.), *Associate Referee*

In the 1951 report it was recommended that the Elmore method for the determination of organic thiocyanate nitrogen in liquid thiocyanate preparations commonly used in livestock and fly sprays be continued as first action pending an investigation of the applicability of the method to ester type thiocyanates. Information at that time showed the Elmore method gave a slightly lower recovery of thiocyanate nitrogen on the ester type thiocyanate, $C_nH_{2n+1}COOCH_2CH_2SCN$ (10–18 carbon atoms), as it occurred in the commercial type product, than that obtained by a Kjeldahl analysis. It was believed this discrepancy was due to the presence of nitrogen in the commercial material in forms other than thiocyanate.

Rohm and Haas Company, a collaborator and manufacturer of ester type thiocyanates, isolated a small amount of β-thiocyano-ethyl ester of capric acid ($C_9H_{19}COOC_2H_4SCN$) by chromatographic techniques. It found 5.12 per cent nitrogen by a Kjeldahl procedure and 5.03 per cent by the Elmore method, and has concluded the analytical discrepancies previously cited are not due to any deficiencies in the Elmore method. It has accordingly withdrawn previous objections to the official adoption of the Elmore method.

RECOMMENDATIONS*

It is recommended that the revised Elmore method for the determination of organic thiocyanate nitrogen in livestock and fly sprays, as published in the 1950 collaborative report (*This Journal*, **34**, 677 (1951)) be adopted as official.

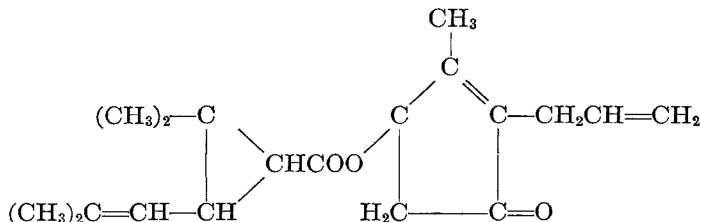
* For report of Subcommittee A and action of the Association, see *This Journal*, 36, 49 (1953).

REPORT ON ALLETHRIN

BY MILTON S. KONECKY (United States Department of Agriculture, Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, Beltsville, Md.), *Associate Referee*

Allethrin is a wholly synthetic product of the same order of toxicity to insects, and as non-toxic to warm-blooded animals, as are pyrethrins. It was synthesized in 1949 by Schechter, Green, and LaForge (1, 2) of the Bureau of Entomology and Plant Quarantine. Allethrin is now being produced on a large scale and has replaced pyrethrins in many insecticide formulations.

Allethrin is an ester of chrysanthemum monocarboxylic acid combined with allethrolone, a cyclic keto-alcohol.



There are eight optical and geometric isomers in technical allethrin. A crystalline isomer, m.p. 50.2–50.6°C., has been separated (3) from the stereoisomeric mixture and is called *alpha-dl-trans*-allethrin. It consists of one of the racemic ester pairs containing the *trans* acid. This crystalline allethrin shows promise as a primary standard for analytical methods and as a reference standard for insecticide test methods. Allethrin is soluble in most organic solvents and practically insoluble in water. Technical allethrin as now produced commercially contains between 75 and 95 per cent allethrin.

Methods of analysis for technical allethrin have not previously been studied by this Association, and this report will therefore review the methods currently in use and under development.

Hydrogenolysis Method.—The hydrogenolysis reaction (4) on which this analytical method is based depends on the cleavage of an ester of a *beta*-unsaturated alcohol to yield free acid, which is then determined by titration. The sample is dissolved in isopropanol and subjected to low-pressure catalytic hydrogenation in a Parr apparatus. Palladium oxide on a barium sulfate carrier is employed as the catalyst. After the catalyst has been removed by filtration, the solution is brought to a boil and titrated, while boiling, with standard alkali. This is called the hot-titration variation. This titration measures the gross allethrin in the sample. Proper corrections for free acidity, catalyst activity, and reagent blanks must be applied to obtain the net allethrin content of the sample.

Recently this method has been modified by adding to the sample before hydrogenation an amount of alkali equivalent to the free acidity of the sample plus one-tenth of a milliequivalent of sodium hydroxide solution in excess. This addition of alkali seems to activate the catalyst, so that the acidity resulting from the cleavage of the ester can be titrated at room temperature instead of at boiling conditions. This modification is called alkali activation. In both of these variations the effectiveness of the catalyst must be established. The anisic acid ester of allethrolone (2-allyl-4-hydroxy-3-methyl-2-cyclopenten-1-one), a solid derivative, is used as a standard for determining catalyst activity. The crystalline *alpha-dl-trans* allethrin is under consideration as a substitute for the allethrolone anisate as a standard for this method.

The total free acidity, the acidity due to free chrysanthemum monocarboxylic acid, and the free chrysanthemum monocarboxylic acid chloride in the allethrin are determined by a potentiometric titration with standard alkali. The corrections for the free acidity and catalyst activity are applied to the gross allethrin as determined above.

The hydrogenolysis method is still in the developmental stage. The presence of chrysanthemum monocarboxylic anhydride in technical allethrin has recently been detected. The effect of this impurity on the hydrogenolysis method has not as yet been fully evaluated. There are some indications that the anhydride interferes quantitatively and therefore can be determined independently and corrected for. Also the presence of small amounts of substances that would poison the catalyst can be a source of trouble in the catalytic hydrogenation method. However, catalyst poisoning has not been experienced in analyzing commercial batches of technical allethrin.

Ethylenediamine Method.—A recently proposed method for the analysis of technical allethrin is the ethylenediamine (5) method, commonly called EDA. In the EDA method allethrin splits to give the ethylenediamine salt of chrysanthemum monocarboxylic acid which titrates as an acid with standard sodium methylate in a pyridine medium. This titration gives the gross allethrin in the sample. Chrysanthemum monocarboxylic acid, its anhydride, and its acid chloride must be determined independently and corrected for to obtain the net allethrin present.

The chrysanthemum monocarboxylic acid and the acid chloride are determined by titration with standard sodium hydroxide in ethanol. The anhydride and the acid chloride are determined by reaction with a measured excess of morpholine followed by titration of the excess morpholine with methanolic hydrochloric acid.

The acid chloride only is found by reacting a sample with methanol and titrating the hydrogen chloride formed with methanolic potassium hydroxide using an indicator whose color change occurs in the correct range of *p*H.

Some details of this method are still under development. A point of

controversy is the choice of conditions to be used for the reaction of the ethylenediamine with the sample. Originally the method provided two alternate conditions: (1) reaction of sample and EDA at 98°±2°C. for ½ hour, and (2) reaction at room temperature for 2 hours. On some samples of technical allethrin these two conditions did not give equivalent results. The conditions for the reaction at 98°C. only were then specified. However, there is now evidence that room temperature (25°±2°C) is preferable.

It is recommended* that the hydrogenation and the ethylenediamine methods of analysis for technical allethrin be further investigated and collaborative studies be initiated.

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REPORT ON PIPERONYL BUTOXIDE

By BOYD L. SAMUEL (Division of Chemistry, Virginia Dept. of Agriculture, Richmond, Virginia), *Associate Referee*

The Associate Referee had expected to carry out collaborative work this year in accord with the recommendations of last year's report on piperonyl butoxide. A partition chromatographic separation followed by a determination based on Jones's (1) color method was tried. However, it was found that samples of piperonyl butoxide from recent production did not behave in the chromatographic column in the same way as the samples on which the preliminary work had been done. A discussion of this development with J. J. T. Graham, the General Referee, ended in agreement that further investigational work should be done before selecting a method for collaborative study.

The Associate Referee recommends† that the study of methods for piperonyl butoxide be continued.

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(1) JONES, H. A., ACKERMANN, H. J., and WEBSTER, M. E., *This Journal*, **35**, 771 (1952).

* For the report of Subcommittee A and action of the Association, see *This Journal*, **36**, 49 (1953).
† For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 49 (1953).

No reports were given on: dimethyl dithiocarbamates; DDT and related compounds; chlordaneand toxaphene; quaternary ammonium compounds; phenolic disinfectants; physical properties of economic poisons; aldrin; and dieldrin.

The contributed paper entitled "Analysis of Manganese Ethylenebisdithiocarbamate—Compositions and Residues" appears on page 482 of *This Journal*.

REPORT ON PLANTS

By E. J. Miller (Michigan Agricultural Experiment Station, East Lansing, Michigan), *Referee*

During the present year several Associate Referees have prepared reports.

Kenneth C. Beeson, Associate Referee on copper and cobalt in plants, submitted a report describing the results of a collaborative study of methods for determining these constituents of plants. Nine collaborators cooperated with the Associate Referee in the study, and the results obtained were in good agreement.

Eunice J. Heinen, newly appointed Associate Referee on sodium in plants, has submitted a report on this subject describing preliminary studies.

Carroll L. Hoffpauir, Associate Referee on starch in plants, reported results from the use of a new adaptation of a method formerly used in the determination of starch in plant materials and found it to give satisfactory reproducibility of results.

Kenneth T. Williams, Associate Referee on sugar in plants, together with Earl F. Potter, reported the results of a collaborative study of two different means of clarifying plant extracts for the determination of sugar therein and a titrimetric method for evaluating micro quantities of dextrose. Results from all collaborators agreed satisfactorily.

E. J. Benne, Associate Referee on zinc in plants, and Eunice J. Heinen reported the results of a collaborative study of a simplified dithizone method for determining zinc in plants which had been described in 1950. There was good agreement in the results from the different collaborators.

No reports were received for boron or carotene.

RECOMMENDATIONS

It is recommended*—

(1) That the Associate Referees on various constituents of plants listed on page 4 of the February 1952 issue of *The Journal* continue with their respective assignments.

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* For report of subcommittee A and action of the Association, see *This Journal*, **36**, 50 (1953).

(2) That the following recommendations of the Associate Referees be accepted:

(a) That the nitroso-R-salt method used in the 1952 collaborative study of the determination of cobalt in plants be adopted, first action.

(b) That the nitroso-cresol method used in the 1949 and 1951 collaborative studies of the determination of cobalt in plants be adopted as an alternative method, first action.

(c) That the sodium diethyldithiocarbamate method for copper in plants studied collaboratively in 1949, 1951, and 1952 be adopted, first action.

(d) That further collaborative work on these methods be postponed since their development is still in progress, but as new methods or modifications appear, additional collaborative work be undertaken.

(e) That the study of methods for the determination of sodium in plants be continued, especially in respect to: (1) A comparison of the values for sodium by the use of the flame photometric and the A.O.A.C. magnesium uranyl acetate procedures in the analysis of a variety of plant materials. (2) Use of the A.O.A.C. method in the analysis of plant tissues which contain only small quantities of sodium. (3) Possible interferences of various ions that commonly occur in plant materials.

(f) That the modified procedure described in the Associate Referee's report for the determination of small amounts of starch in plant materials be submitted to collaborative study.

(g) That the micro method for dextrose be adopted, first action.

(h) That the collaborative study of the ion-exchange method of clarifying solutions for the determination of sugar be continued.

(i) That the study of methods for determining zinc in plant materials be continued.

(3) That an Associate Referee be appointed to study flame photometric procedures for the determination of potassium in plants, with the objective of including such a method in the 1955 issue of *Methods of Analysis*, A.O.A.C.

ACKNOWLEDGMENT

The General Referee wishes to express his appreciation to the Associate Referees for their accomplishments and cooperation during the past year.

REPORT ON SODIUM IN PLANTS*

By EUNICE J. HEINEN (Michigan Agricultural Experiment Station, East Lansing, Michigan), *Associate Referee*

The present A.O.A.C. gravimetric method (1) for the direct determination of sodium in plant materials depends upon its precipitation as the

* Published with the approval of the Director of the Michigan Agricultural Experiment Station as *Journal Article* No. 1934.

triple salt of sodium magnesium uranyl acetate. This method, an adaptation of the method developed by Caley and Foulk (2), was accepted by the Association in 1935 as a tentative procedure.

In 1948, Shirley was appointed as Associate Referee on sodium in plants. His work on this subject has been published in *This Journal* in 1949 (3) and in 1950 (4). The results of the study published in 1949 (3) were centered around a comparison of the magnesium uranyl acetate and zinc uranyl acetate procedures, and the precision of the former. In 1950, Shirley and Benne (4) published the results of a collaborative study involving the use of the flame photometer for the determination of sodium in plant materials. The values thus obtained were compared with those from the gravimetric magnesium uranyl acetate method. At that time, the Associate Referee recommended that the tentative A.O.A.C. method be made official, first action, and that the study of methods for determining sodium in plant materials be continued, especially in respect to the use of the flame photometer.

Following the resignation of Shirley, the present Associate Referee was appointed late in 1951 to continue the study of methods for the determination of sodium in plant materials. Little opportunity has been available since then to carry out an extensive investigation of the subject; however, work done was centered around the following:

(A) A review of the copious literature on this subject.

(B) A limited study of the use of the flame photometer, and a comparison of the values for sodium obtained by its use with those by the A.O.A.C. method.

(C) A brief investigation of factors which affect the precision of results from the magnesium uranyl acetate method.

A. LITERATURE REVIEW

It is not feasible to attempt a complete review of literature on the subject in a report of this kind. Interested readers are referred to Collins' (5) 40-page article (1943), which contains 187 references to articles pertaining to methods for determining sodium. Only a few recent articles are cited here.

In general, the chemical methods for the direct determination of sodium depend upon the precipitation of the triple salt formed between sodium and the uranyl acetates of magnesium, zinc, manganese, nickel, or cobalt. Many modifications and adaptations of these procedures have been developed, including numerous attempts to utilize such triple salts for evaluating sodium colorimetrically rather than gravimetrically. An example of this is the recently published work of Stone and Goldzieher (6) who have developed a colorimetric method designed for determining sodium in biological fluids, particularly in serum. In this procedure the sodium is precipitated as sodium zinc uranyl acetate, and treated in an alkaline solution with hydrogen peroxide to produce an intensely reddish-

yellow colored complex, which is evaluated photometrically. This color is reported to be stable for two hours and to be unaffected by variations in temperature between 20° and 30°C. The complete procedure is claimed to have an accuracy of approximately 1 per cent.

Pisha and Speier (7) have published a method depending upon the formation of sodium zinc uranyl acetate. The triple salt is precipitated in a carbon dioxide-freezing chamber, followed by mechanical shaking of the partially frozen solution in which the ice particles act as efficient stirrers. The precipitation is complete within ten minutes. The salt is filtered, dissolved, and evaluated photometrically, the accuracy of the method being within 0.5 per cent.

In recent years much interest has centered around the determination of sodium in plant materials by use of the flame photometer. Attoe (8); Toth, Prince, Wallace, and Mikkelsen (9); and Seay, Attoe, and Truog (10) have published papers dealing with this subject.

B. COMPARISON OF SODIUM VALUES

Several different plant tissues were analyzed for sodium by the magnesium uranyl acetate procedure and by the Perkin-Elmer flame photometer, Model 52A, using acetylene gas as fuel. In preparing the sample for the flame photometer, a weighed portion of plant material was ashed with H_2SO_4 in a muffle furnace at approximately 500°C., the ash was digested with HCl, and the calcium was precipitated as the oxalate as directed in *Methods of Analysis*, **6.12**. The silica and calcium oxalate were then removed by filtering into a volumetric flask, and sufficient standard lithium chloride solution was added so that the solution, when made to volume, would contain 25 p.p.m. of lithium as an internal standard. The results obtained are given in Table 1.

TABLE 1.—*Per cent* sodium determined by different methods*

PLANT TISSUE ANALYZED	MAGNESIUM URANYL ACETATE METHOD	FLAME PHOTOMETER METHOD
Alfalfa hay no. 1	.061	.068
Alfalfa hay no. 2	.042	.045
Apple leaves	.025	.032
Cherry leaves	.032	.025
Citrus leaves	.067	.074
Peach leaves	.010	.017

* Averages of replicate results.

C. ACCURACY OF THE SODIUM MAGNESIUM URANYL ACETATE METHOD

A review of the literature revealed that investigators have differed greatly as to the length of time recommended for complete precipitation

TABLE 2.—*Effect of time on completeness of precipitation of sodium magnesium uranyl acetate*

SODIUM PRESENT	PRECIPITATED FOR 2 HOURS AT 22°C.		PRECIPITATED FOR 4 HOURS AT 22°C.	
	SODIUM RECOVERED		SODIUM RECOVERED	
mg	*mg*	*per cent*	*mg*	*per cent*
0.6	0.35	58.3	0.38	63.3
1.0			0.86	86.0
5.0	4.96	99.2	5.01	100.2

of the triple salt. Such recommendations varied from a 2 to 3 minute period of vigorous stirring to an hour of stirring by motor-driven apparatus, and sometimes included a period of a day or more of standing. The author attempted to determine if a 3 minute period of vigorous stirring, followed by several hours in a water bath at 22°C. with occasional additional stirring, would result in complete precipitation of the sodium salt and thereby shorten the 24 hour period prescribed in the official method. Appropriate aliquots of a standard solution of sodium chloride were placed in beakers and made up to 5 ml with distilled water; 100 ml of magnesium uranyl acetate reagent was added, the solutions were vigorously stirred for 3 minutes, and placed in a water bath at 22°C. for the remainder of the precipitation period. The solutions were stirred occasionally throughout this interval of time.

The time intervals used and the results obtained are given in Table 2.

While analyzing certain plant tissues for their sodium content with the A.O.A.C. procedure, it was noted in some instances that crystals separated out upon concentration of the solution to 5 ml. Therefore the effect on the sodium values when solutions were concentrated only to 10 ml was investigated. Aliquots of a standard sodium chloride solution were placed in beakers and distilled water was added to bring them to 10 ml. A 100 ml portion of the magnesium uranyl acetate reagent was added to each, the solutions were stirred vigorously for 3 minutes and left in a water bath at 22°C. for 4 hours with occasional stirring.

The results obtained are given in Table 3.

TABLE 3.—*Effect of volume of solution on completeness of precipitation of sodium magnesium uranyl acetate during a 4 hour period in a water bath at 22°C.*

SODIUM PRESENT	VOL. OF 5 ML.		VOL. OF 10 ML.	
	SODIUM RECOVERED		SODIUM RECOVERED	
mg	*mg*	*per cent*	*mg*	*per cent*
0.6	0.38	63.3	0.26	43.3
1.0	0.86	86.0	0.57	57.0
5.0	5.01	100.2	4.84	96.8

DISCUSSION

The data in Table 1 show that the values for sodium by the flame photometer agree fairly well with those obtained by the direct A.O.A.C. method. However, in most cases the magnesium uranyl acetate values are somewhat lower, possibly due to incomplete precipitation of the triple salt. Hence the Associate Referee feels that this study should be extended to include a greater variety of plant tissues, and the completeness of precipitation of the triple salt should be investigated further.

The data in Table 2 indicate that a four hour period of precipitation, following three minutes of vigorous stirring, is insufficient to precipitate completely small quantities of sodium, although this period of time is sufficient for larger quantities of sodium, such as 5 mg. Caley (11) has done considerable work on the determination of minute amounts of sodium by the magnesium uranyl acetate method, and his work shows that this method can be used satisfactorily on solutions containing as little as 0.2 mg of sodium. It is noted, however, that he used vigorous mechanical stirring for a one hour period of time to insure complete precipitation. The Associate Referee therefore believes that the period of vigorous stirring, or the period of precipitation, or both, must be extended beyond that carried out in this particular experiment to precipitate completely small quantities of sodium.

The data in Table 3 illustrate the importance of concentrating the volume of the solution to 5 ml or less before the addition of the magnesium uranyl acetate reagent. As previously noted, however, the author had difficulty with the separation of crystals when some solutions were concentrated to this volume. If a white precipitate of $CaSO_4$ forms at this time, Caley and Foulk (12) recommend the addition of 0.3–0.5 gram of solid ammonium chloride and shaking until the precipitate dissolves. This treatment was tried and the crystals were brought into solution in all cases except for one sample. It was hoped in this case that the crystals would dissolve in the reagent, but after vigorous stirring and a five-hour period of precipitation in a water bath at 22°C., crystals still persisted. When the temperature was raised to 33°C. for 2 hours, however, and upon occasional stirring, they dissolved and did not re-precipitate either at 22°C. or at 4°C. during the remainder of the 24 hour precipitation period.

RECOMMENDATIONS*

It is recommended that the study of methods for the determination of sodium in plants be continued, especially in respect to:

1. A comparison of the values for sodium obtained by use of the flame photometric and the A.O.A.C. magnesium uranyl acetate procedures in the analysis of a variety of plant materials.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 51 (1953).

2. Use of the A.O.A.C. method in the analysis of plant tissues which contain only small quantities of sodium.

3. Possible interferences of various ions that commonly occur in plant materials.

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REPORT ON ZINC IN PLANTS*

BY EUNICE J. HEINEN and ERWIN J. BENNE, *Associate Referee* (Michigan Agricultural Experiment Station, East Lansing, Michigan),

A study of methods for the determination of zinc in plants has been in progress in the authors' laboratory for a number of years. Reports on the subject have included that of Cowling in 1941 (1), those of Shirley, *et al.*, in 1948 (2) and 1949 (3), and those of Heinen and Benne in 1951 (4) and 1952 (5). In 1941 Cowling (1) recommended that a mixed-color dithizone method be included in *Methods of Analysis*, A.O.A.C., and the successive studies have largely represented efforts to simplify this procedure without loss of accuracy.

Shirley, *et al.*, in 1948 (2) and again in 1949 (3) reported results obtained by using the dithizone method as a one-color procedure and compared them with values from the mixed-color A.O.A.C. procedure. In 1951 Heinen and Benne (4) described a simplified one-color dithizone procedure which gave results for zinc in good agreement with those by the official method. Consequently, the Associate Referee recommended that this simplified procedure be studied collaboratively, and such a study was conducted during the present year. Five laboratories were requested to

* Published with the approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 1395.

participate. Four agreed to do so and were furnished 3 samples to analyze for zinc by the simplified procedure (4) and by any other method they wished to use. Of the samples furnished, No. 1 was oat grain, No. 2 was mixed hay, and No. 3 was wheat middlings.

Three of the collaborating laboratories reported results in time to be included in this report. The authors gratefully acknowledge their cooperation. The names and locations of the individuals who participated in the study are as follows:

No. 1. W. R. Flach, Eastern States Farmers' Exchange, Inc., Buffalo, New York.

No. 2. John W. Kuzmeski and C. T. Smith, University of Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.

No. 3. Fred E. Randall, Cooperative G. L. F. Exchange, Inc., Buffalo, New York.

No. 4. The authors.

The results obtained by the collaborators and the procedures used are summarized in the table which follows:

TABLE 1.—*P.p.m. of zinc*[1] *expressed on the air-dry basis*

COLLABORATOR	SIMPLIFIED PROCEDURE			OTHER PROCEDURES[2]		
	SAMPLE NO.			SAMPLE NO.		
	1	2	3	1	2	3
No. 1	29	20	100	30	28	104
No. 2	26	19	90	30	23	103
No. 3	29	20	97	—	—	—
No. 4	30	21	96	30	23	101
Averages	29	20	96	30	25	103

[1] Averages of results from replicated determinations.
[2] A.O.A.C. mixed-color procedure except for collaborator No. 2 whose procedure is described under his comments.

COMMENTS OF COLLABORATORS

No. 1.—We had no difficulty with either procedure, and certainly the results are very encouraging.

No. 2.—We have determined the zinc by the proposed method, and are far from satisfied with our results. The main difficulty was the excessive blank, amounting to 1 part in about 3.5 parts of final zinc measured in the samples low in zinc. This may be due to the sulfuric acid (difficult to purify) used in the ashing, or to the reaction between this acid and the porcelain crucibles which are hard to clean. We had no difficulty in this regard when the determinations were repeated by the A.O.A.C. method for zinc in Chapter 24, "Metals, Other Elements and Residues in Foods," where we simply ashed the sample at about 600° and obtained the negligible blank for this type of work of about 1 part in 40. The sulfuric acid ashing should, in our opinion, be omitted as it introduces more errors than it eliminates. The work of Heinen and Benne themselves indicates that it is unnecessary. We believe that the simple directions recently used in a collaborative study of cobalt by the Florida

method are applicable for most trace metals: viz., "Ash 2 g sample for 2 hrs at 600°C. Transfer to a 200 ml volumetric flask with 20.0 ml HCl and 50 ml H_2O, boil 5 min., cool, make to mark, allow to settle and pipet a suitable aliquot." (For exact work, repurified HCl may be used.)

A second possible source of error in the proposed method seems to be in the elimination of Cu by dithizone in an unbuffered solution, by controlling the *p*H with various specified amounts of acids and alkalies of various specified normalities. Small errors here can lead to large errors in the final *p*H. All our experience indicates that it is more accurate and much simpler to eliminate Cu from a citrate buffered solution, where the proper *p*H is controlled by an internal indicator, of which there are several available. We prefer the method given for the isolation of Cu by **24.23**. Since Cu was present in the three samples submitted in only very small amounts, we wonder how the proposed method would work in those cases where the Cu present exceeded the Zn.

In the final measurement of zinc, using our Coleman spectrophotometer, the colors were still so intense that we obtained an unreasonably steep curve (not a straight line) and the higher values were beyond the reliable range of the instrument. It was necessary to make our readings against the blank standard instead of against CCl_4 as given in the method. Also, the 0.01 N NH_4OH failed to remove all the excess dithizone, so that we actually had colors ranging from green through the various shades of violet. We prefer the old method of dilution of the final solutions, and believe this step should be left to the analyst, who is best acquainted with the machine available to him. We generally carry up to 40 mmg of zinc through the procedure, and make the final dilution of sample and standards just before reading. With this procedure we have experienced no difficulty from fading, as the stronger solutions appear to be more stable, even though our separatory funnels are plain pyrex. We work in diffused light.

We have repeated the work by our normal procedure, which consists of removal of Cu as dithizonate instead of as sulfide, then followed the procedure for zinc as given in the chapter on "Metals, Other Elements and Residues in Foods," chapter 24. We consider that these values more accurately represent the true amount of zinc present.

We cannot see the necessity for a special method for zinc in plants, as we believe that the method for Zn in Chapter 24 already supplies the need, although this method could be greatly shortened. This latter method is highly flexible and can readily be adapted to plants, grain and stock feeds, mineral supplements, etc. We feel that further work can be done to combine the best features of each method, and so to shorten the procedure. To this end, we suggest (1) that Cu be eliminated in a form where it can be measured if desired, as dithizonate, by **24.23**; (2) that the elimination of Co and Ni by **24.99** be discontinued, and that instead, the zinc dithizonate be isolated directly in the presence of carbamate solution, as in the present proposed collaborative procedure; (3) that the final Zn be measured by the technique of **24.100**, with possibly some improvements.

DISCUSSION

From the foregoing table it is evident that the values for zinc obtained by the different collaborators with the simplified procedure agree well for a given sample, and that they in turn are in good agreement with the results from the other procedures used. Replicate results reported by collaborators for a given sample generally agreed so well it seemed unnecessary to include more than average values.

It is of interest that the extremes of digression from the average values obtained for a given sample by the different collaborators with the simplified procedure were no greater than were those among results obtained with the other procedures used. However, even though the results of this collaborative study are encouraging, it would seem of value to attempt to improve the procedure still further, if possible, perhaps along the lines suggested in the comments of collaborator No. 2. Therefore, it is recommended* that the study be continued.

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REPORT ON STARCH IN PLANTS

MODIFICATION OF THE ANTHRONE PROCEDURE

By Carroll L. Hoffpauir (Southern Regional Research Laboratory, New Orleans, Louisiana), *Associate Referee*

A preliminary investigation of the application of the anthrone-sulfuric acid reagent in the final evaluation of starch isolated from plant materials was described in the previous report (1). When the procedure was applied to plant materials, the values obtained showed such poor reproducibility that they were completely unsatisfactory. It seemed likely that the erratic results were due to losses during the purification of the starch. Recovery of starch at each step of the procedure was investigated, using peanut meal and buckwheat leaves from which sugars had been extracted with alcohol so that the anthrone-sulfuric acid reaction could be applied to the extracted starch at each step of the purification procedure. This investigation indicated that the final dispersion of the purified starch prior to color development was incomplete, while satisfactory recoveries were obtained in each of the other steps involved.

Several reagents were tried for dispersing the purified starch, including potassium hydroxide, calcium chloride, and perchloric acid. The most satisfactory procedure was as follows:

After the final washing with alcoholic sodium chloride as described in the previous report (1), add 5 ml of 4.8 N perchloric acid to the purified starch. Place the tube in an H_2O bath at 25°C. and stir frequently for 30 min. Transfer to a 250 ml volumetric flask, make to vol. with H_2O, and

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 50 (1953).
† One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

mix well. Determine the starch in a 5 ml aliquot exactly as described previously (1).

Samples of alfalfa, buckwheat leaves, and peanut meal were analyzed by the modified procedure by three analysts. The results, shown in Table 1, indicate satisfactory reproducibility for the method. A series of 6 determinations using a sample of sweet potato starch of known purity gave recoveries which ranged from 96.6 to 101.0 and averaged 99.0 per cent.

The modified procedure described above shows considerable promise for the determination of starch occurring in small amounts in plant materials and should be submitted to collaborative study.

TABLE 1.—*Starch content of alfalfa, buckwheat leaves, and peanut meal (moisture-free basis)*

ANALYST	ALFALFA	BUCKWHEAT LEAVES	PEANUT MEAL
	per cent	*per cent*	*per cent*
1	0.24	11.3	5.8
	0.26	11.3	5.7
		11.6	
Average	0.25	11.4	5.75
2	0.27	11.2	6.0
	0.18	11.1	5.8
Average	0.23	11.15	5.9
3	0.27	10.7	5.2
	0.17	10.8	5.2
			5.2
Average	0.22	10.75	5.2

ACKNOWLEDGMENT

The Associate Referee wishes to thank Miss E. R. McCall and Mr. L. E. Brown for some of the analyses reported.

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REPORT ON SUGARS IN PLANTS

BY KENNETH T. WILLIAMS, *Associate Referee*, and EARL F. POTTER (Western Regional Research Laboratory,* Albany, California)

For several years the Associate Referee has been studying methods of clarification of plant extracts for sugar analysis. During this study it was

* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U.S. Department of Agriculture.

found that certain ion-exchange resin combinations were as good as, or better than, lead acetate for removing the non-sugar reducing materials from the plant extracts. Progress reports have been made (*This Journal*, **33**, 816 (1950); **34**, 700 (1951); **35**, 402 (1952)).

During the past year the ion-exchange method has been compared with the official lead acetate method by a limited number of collaborators. The results are very encouraging and it is hoped that additional collaborators will be interested.

METHOD

MATERIALS FURNISHED COLLABORATORS

The plant extracts prepared with hot 80% alcohol.
Cation exchange resin, Amberlite IR-100 (H) AG.[1]
Anion resin, Duolite A5.
Celite analytical filter aid.
U. S. Bureau of Standards dextrose.

INSTRUCTIONS TO COLLABORATORS

Measure accurately the suggested aliquot into a beaker and evap. on the steam bath to drive off alcohol. Avoid evapn to dryness by adding H_2O. When odor of alcohol has disappeared, add about 15–25 ml of H_2O and heat to 80°C. to soften gummy ppts and break up insoluble masses. Cool to room temp. Prepare a thin mat of Celite on a filter paper in a Büchner funnel or on a sintered glass filter and wash until H_2O comes through clear. Now filter sample through Celite mat, wash mat with distd H_2O and make filtrate and washings to the suggested vol. in a volumetric flask. Mix well and label soln A.

Place a 25.0 ml aliquot of soln A (run at least 2 replicates) in a 250 ml Erlenmeyer flask, add 25.0 ml of distd H_2O, and then add 2 g of IR-100 cation and 3 g of A4 anion ion-exchange resins. Allow to stand 2 hrs with occasional swirling. Take a 5 ml aliquot of the de-ionized soln and det. sugar as directed under **29.61**, **29.62**, and **29.63** of *Methods of Analysis*, 7th Ed. (1950).

Transfer a 25.0 ml aliquot of soln A to a 50 ml volumetric flask. Add 1 ml satd neutral lead acetate soln to produce a flocculent ppt, shake thoroly, and allow to stand 15 min. Test supernatant liquid with a few drops of the lead acetate soln. If more ppt forms, shake, and allow to stand again; if no further ppt forms, dil. to mark with H_2O, mix thoroly, and filter thru dry filter paper, discarding the first few ml of filtrate. Add sufficient solid sodium oxalate to filtrate to ppt all the Pb, and refilter through a dry paper, discarding the first few ml of filtrate. Test filtrate for presence of Pb with a little sodium oxalate; refilter as above if more ppt forms. Take a 5 ml aliquot of this clarified soln and det. sugar as directed under **29.61**, **29.62**, and **29.63** of *Methods of Analysis*, 7th Ed. (1950).

RESULTS

For the first collaborative study of the ion-exchange method of clarification, four very different plant materials were chosen. The experiment was designed to give a direct comparison of the two methods of clarification. All of the data submitted by the collaborators are given in Table 1.

[1] Mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others not mentioned.

TABLE 1.—*Results expressed as mg of dextrose in 5 ml aliquot of clarified solution taken for the determination of sugar*

COLLABORATOR	CLARIFIED WITH LEAD		CLARIFIED WITH ION-EXCHANGE RESINS	
		Crab Apple		
A[1]	1st[2]	0.99, 1.01	1st[2]	1.00, 1.01
	2nd	1.00, 1.01	2nd	1.02, 1.03
B[1]	1st	0.96, 1.00	1st	1.03, 1.01
E. F. Potter[1]	1st	0.96	1st	0.96
	2nd	0.96	2nd	0.95
C	1st	1.13, 1.09, 1.05	1st	1.00, 1.01, 1.03
	2nd	1.08, 1.10	2nd	1.09, 1.05
D[1]	1st	1.17, 1.15	1st	1.19, 1.21
	2nd	1.19, 1.16	2nd	1.19, 1.20
		Mustard Greens		
A	1st	1.12, 1.10	1st	0.99, 1.02
	2nd	1.07, 1.07	2nd	0.99, 0.98
B	1st	1.01, 1.04	1st	0.99, 1.04
E. F. Potter	1st	1.10	1st	0.96
	2nd	1.10	2nd	0.96
C	1st	1.12, 1.15	1st	0.94, 0.92
	2nd	1.22, 1.13, 1.09	2nd	0.91, 0.91
	3rd	1.13, 1.14	3rd	1.00, 1.03
D	1st	1.10, 1.11	1st	1.02
	2nd	1.10, 1.09	2nd	1.01, 1.01
		Dehydrated Potato		
A	1st	1.10, 1.12	1st	0.94, 0.97
	2nd	1.13, 1.13	2nd	0.99, 0.98
B	1st	1.16, 1.16	1st	1.04, 1.03
E. F. Potter	1st	1.13	1st	0.96
	2nd	1.15	2nd	0.96
C	1st	1.22, 1.22	1st	1.00, 1.03, 1.00
	2nd	1.16, 1.28, 1.22	2nd	0.95, 1.06, 0.99
	3rd	1.21, 1.23	3rd	1.00, 1.00
D	1st	1.19, 1.14	1st	1.08, 1.06
	2nd	1.18, 1.12	2nd	1.04, 1.04
		Peach Leaf		
A	1st	1.15, 1.15	1st	1.11, 1.11
	2nd	1.18, 1.17	2nd	1.09, 1.09
B	1st	1.12, 1.12	1st	1.19, 1.22
E. F. Potter	1st	1.15	1st	1.08
	2nd	1.13	2nd	1.08
C	1st	1.24, 1.26, 1.22	1st	1.14, 1.14
	2nd	1.24, 1.21, 1.23	2nd	1.13, 1.14, 1.13
	3rd	1.26, 1.22, 1.25		
D	1st	1.10, 1.14	1st	1.09, 1.11
	2nd	1.13, 1.09	2nd	1.09, 1.10

[1] Values taken from a standard curve prepared by the collaborator.
[2] Aliquot of A.

On the assumption that no reducing sugars are destroyed or removed by ion-exchange resins, the data indicate that the resins remove non-sugar reducing substances better than lead acetate does. The results in Table 1 are lower and more consistent using ion-exchange as compared with lead acetate clarification. However, tests must be made on a much larger variety of samples by a larger number of collaborators.

COMMENTS BY COLLABORATORS

Collaborator A.—(1) The use of ion-exchange resins as clarifying agents requires less manipulation than does the use of the lead ion for that purpose. (2) Trials with known quantities of dextrose showed no loss due to adsorption on the resins. (This is a needed confirmation of the data obtained by the Associate Referee, *This Journal*, **33**, 987–994 (1950).) (3) Waiting for 2 full minutes after adding the sulfuric acid before beginning to titrate gave erratic results in some cases; hence, the practice of beginning to titrate immediately after the cuprous oxide was dissolved was adopted.

Collaborator B.—The Celite filtration was found to be a bit slow with the peach leaf extract.

COLLABORATORS

There was 100 per cent participation by collaborators as follows (the order has no bearing on the letter designations used above): Mary E. McKillican, Department of Agriculture, Ottawa, Ontario; E. J. Benne and Eunice Heinen, Michigan State College, East Lansing, Michigan; H. L. Wilkins, Department of Agriculture, Beltsville, Md.; Fred E. Randall, Cooperative Grange League Federation Exchange, Inc., Buffalo, New York.

RECOMMENDATIONS

In the chapter on Plants, in the 1950 Edition of *Official Methods of Analysis*, two methods are referred to under reducing sugars. The references are to Munson-Walker General Method (see **29.36**) and to Quisumbing-Thomas Method (see **29.47**). These methods are not applicable when the amount of sugar available for analysis is small, for example, 20 mg. In the chapter on Sugar and Sugar Products there is also a micro method for dextrose (see **29.61, 29.62,** and **29.63**) that is very good when the amount of sugar is limited.

It is recommended*—

(1) That the micro method for dextrose be adopted, first action.

(2) That the collaborative study of the ion-exchange method of clarification be continued.

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 50 (1953).

REPORT ON COPPER AND COBALT IN PLANTS

By KENNETH C. BEESON (U. S. Plant, Soil, and Nutrition Laboratory, Bureau of Plant Industry, Soils, and Agricultural Engineering, A.R.A., U. S. Department of Agriculture, Ithaca, New York), *Associate Referee*

It was recommended at the 1951 meeting of the Association that collaborative studies of methods for the determination of cobalt and copper in plants be continued and that the nitroso-R-salt reagent for cobalt be used in place of nitroso-cresol (1).

The sodium salt of 1-nitroso-2-hydroxynaphthalene-3,6-disulfonate commonly known as nitroso-R-salt was first used by Van Klooster (2) to determine cobalt. Using this reagent Stare and Elvehjem (3) developed a method for cobalt in biological materials that was subsequently greatly improved by McNaught (4) and many other workers. The method used in this study is essentially that of McNaught but with several modifications used in the laboratories of collaborators and the Associate Referee.

COLLABORATORS

Twelve analysts were each sent the three samples with instructions for determining cobalt and copper. One of these collaborators, M. T. Mathis (Referee on spectrographic methods) requested the samples in order to compare spectrographic with colorimetric methods. Ten of the collaborators and Mr. Mathis reported on their analyses. Those reporting are as follows:

1. W. B. Deijs, Centraal Instituut voor Landbouwkundig Onderzoek, Wageningen, Netherlands.
2. W. R. Flach, Eastern State Farmers Exchange, Buffalo, New York.
3. Richard L. Gregory, U. S. Plant, Soil and Nutrition Laboratory, Ithaca, New York.
4. F. B. Johnston (R. B. Carson), Plant Chemistry Unit, Division of Chemistry, Department of Agriculture, Ottawa, Canada.
5. I. Motzok, Department of Animal Nutrition, Ontario Agricultural College, Guelph, Canada.
6. Nelson O. Price, Department of Agricultural and Biological Chemistry, Virginia Agricultural Experiment Station, Blacksburg, Virginia.
7. Fred E. Randall, Mills Division, Cooperative Grange League, Federation Exchange, Inc., Buffalo, New York.
8. H. C. Wolf, Testing Laboratory, Kellogg Company, Battle Creek, Michigan.
9. W. T. Mathis, The Connecticut Agricultural Experiment Station, New Haven, Connecticut.
10. C. Tyson Smith, Massachusetts Agricultural Experiment Station, Amherst, Massachusetts.
11. Maurice M. Phillippe, The Clemson Agricultural College, Clemson, South Carolina.

Three samples of plant material, alfalfa, timothy, and buckwheat flour, representing a normal range of cobalt and copper contents, were sent each collaborator. The alfalfa and timothy were from the same lots as the 1951 samples. The buckwheat was a new lot of material. The alfalfa and timothy were ground in a Wiley mill to pass a 20 mesh screen. The buckwheat flour, of course, was already of sufficient fineness for sampling.

The directions for the preparation of reagents (except those specifically used for the nitroso-cresol method), special equipment, and cleaning of glassware were exactly as described by Gregory, Morris, and Ellis (5) and used in the 1951 collaborative study (6) of cobalt determinations.

DIRECTIONS FOR ANALYSIS

The following directions were given for the preparation of additional reagents required for the nitroso-R-salt method for cobalt:

(a) *Nitroso-R-salt.*—0.2%. Dissolve 2 g powd. nitroso-R-salt (Eastman Kodak Company) in redistd H_2O and dil. to 1 l.

(b) *Nitric acid solution.*—(1+1). Dil. concd HNO_3 with an equal vol. distd H_2O and redistill in an all pyrex app. Store in pyrex bottles.

(c) *Bromine water.*—A satd soln of Br in redistd H_2O.

(d) *Citric acid.*—0.2 *N*. Use special reagent grade Pb-free citric acid.

The following directions for additional reagents required for the carbamate method for copper were sent each collaborator:

(a) *Sodium diethyldithiocarbamate.*—0.1% soln. Freshly prepd in redistd H_2O.

(b) *Copper standard soln.*—Dissolve 0.3930 g $CuSO_4 \cdot 5H_2O$ in redistd H_2O, add 5 ml H_2SO_4 and make up to a l and mix. Take 10 ml aliquot, add 5 ml H_2SO_4 and make up to a l and mix. 1 ml contains 1 mmg Cu.

PREPARATION OF SAMPLE

The directions of Gregory, Morris, and Ellis (7) were followed.

For the nitroso-R-salt method it is necessary to take a larger sample for analysis than for the nitroso-cresol method. A suitable wet-digestion method is satisfactory and was used by some collaborators. The following dry-ashing procedure was recommended:

Weigh 10 g dry plant tissue into a clean Pt dish. Cover with pyrex watch glass, and place in cool muffle; heat slowly to 500°C. for overnight. Remove sample and cool. Wet down ash carefully with fine stream of redistd H_2O. From dispensing buret add slowly 2–5 ml $HClO_4$, dropwise at first to prevent spattering. Add ca 5 ml H_2F_2. Evap. on steam bath. Transfer to sand bath and maintain at medium heat until fuming ceases. Cover with pyrex watch glass and return to the partially cooled muffle and heat gradually to 600°C. Allow to remain at this temp. for one hr. Remove sample and cool. Add 5 ml 1+1 HCl and ca 10 ml redistd H_2O. Replace cover glass and warm on steam bath to effect soln. Usually a clear soln essentially free of insol. material is obtained.

DITHIZONE EXTRACTION

The nitroso-R-salt procedure differs at this point from the nitroso-cresol method in that the entire 10 g sample as ashed must be used.

Also the residue from the dithizone extract is dissolved in 0.2 *N* citric acid. The directions given the collaborators follow:

Transfer the soln to a 120 ml separatory funnel (use vaseline as stopcock lubricant). Add 5 ml 40% NH_4 citrate soln. Add one drop phenolphthalein and adjust to *p*H 8.5 with 1+1 NH_4OH. If ppt forms add addnl NH_4 citrate. Add 10 ml dithizone in CCl_4 and shake 5 min. Draw off CCl_4 phase into 100 ml beaker. Repeat as many times as necessary using 5 ml quantities of dithizone soln and shaking for 5 min. each time. The extn is complete when aq. phase remains orange and CCl_4 phase remains predominantly green in color. Then add 10 ml CCl_4, shake 5 min., and combine with CCl_4 ext. The final 10 ml CCl_4 should be pure green. If not, extn was incomplete and must be repeated. Add 2 ml $HClO_4$ to combined CCl_4 exts, cover beaker with pyrex watch glass, and digest on hot plate until colorless. Remove cover glass and evap. slowly to dryness. If sample is heated for any length of time at a high temp. after coming to dryness, losses of Co may occur. Heat only enough to completely evap. to dryness. If free acid remains it will interfere with the next step where *p*H control is important. Dissolve in 1 ml 0.2 *N* citric acid, transfer to 25 ml volumetric flask, and make to vol. with redistd H_2O.

DETERMINATION OF COBALT

Transfer suitable size aliquot of the citric acid soln (ca 8 g dry material) to 50 ml beaker. Evap. to 1–2 ml. Add 3 ml Na borate buffer and adjust *p*H to 8.0–8.5 with NaOH (check externally with phenol red). Vol. should not exceed 5 ml. Add 1 ml of nitroso-R-salt soln *slowly with mixing*. Boil 1–2 min. Add 2 ml 1+1 HNO_3. Boil 1–2 min. Add 0.5–1.0 ml satd $Br\text{-}H_2O$, cover with watch glass and let stand warm for 5 min. Boil 2–3 min. to remove excess Br.

Cool and make up to 10 or 25 ml (depending on length of light path in absorption cell). Transfer to absorption cell and read at 500 mμ within an hr. Standards containing 0.5, 1, 2, 3 and 4 mmg Co should be carried through the same procedure as for the unknown beginning with "Determination of cobalt."

DETERMINATION OF COPPER

The directions for determining copper sent the collaborators are essentially the same as presented in the report of 1950 (8). However, the method was reworded in the interests of clarity and is herewith presented in full:

Transfer an aliquot (1 g dry material) from the citric acid soln obtained from the section, *Dithizone Extraction, nitrosocresol method,* to 125 ml separatory funnel. Add 2 ml NH_4 citrate and 1 drop phenolphthalein. Add 5 ml of Na diethyldithiocarbamate soln. Add NH_4OH (1+1) until pink. Add 10 ml CCl_4. Shake 5 min. Draw off the CCl_4, centrifuge for 5 min. Transfer to an absorption cell and read with filters (Corning) 3389 and 5113, or at 430 mμ.

Prep. a standard curve with 1, 5, 10, 15 and 20 mmg Cu treated as in the *Determination of Copper.*

RESULTS OF THE COLLABORATIVE STUDY ON COBALT

The results of the analyses, presented in Table 1, are on the whole, in fairly good agreement. The largest deviation from the mean among the alfalfa values is 0.04 p.p.m. and the average deviation is 0.01 p.p.m. In the Associate Referee's laboratory an agreement within 0.01 p.p.m.

between two analyses of the same sample is considered eminently satisfactory.

Collaborators 7 and 8 reported rather high results for cobalt on both the timothy and buckwheat samples. In each case the average deviation, for all samples, is 0.02 p.p.m. which is not excessive. The extreme deviation is rather large, however, for the buckwheat samples. Omitting the results of 7 and 8 reduces the mean for buckwheat to 0.03 p.p.m. and the average deviation from the mean to 0.01 p.p.m. The deviations of the results of 7 and 8 from the adjusted mean are then 0.07 and 0.06 p.p.m., respectively, which are greater than would be expected from a consideration of normal probability. Applying the same reasoning to the timothy samples reduces the mean to 0.04 and the average deviation to 0.01, but only the result of collaborator 8 falls outside the limits of probability.

In the collaborative study reported in 1951, using the nitroso-cresol method, the mean value for alfalfa was 0.15 p.p.m. of cobalt and for timothy 0.05 p.p.m. Dr. Johnston determined cobalt on the 1952 samples by the α-nitroso-β-napthol method with the following results: alfalfa 0.14 p.p.m., timothy 0.06 p.p.m., and buckwheat flour 0.05 p.p.m. These results are all in very close agreement and show that any of these methods will give comparable results. However, it should be noted that from 3 to 4 times as much material is required for the nitroso-R-salt method as for the nitroso-cresol method. Thus, the latter method has this advantage, particularly as a research tool.

RESULTS OF THE COLLABORATIVE STUDY ON COPPER

The results reported by the collaborators on the copper content of the three samples are also presented in Table 1. The average deviations from the means are as follows: alfalfa 0.9 p.p.m., timothy, 0.5 p.p.m. and buckwheat 0.4 p.p.m. If the result reported by 8 is eliminated from the alfalfa samples the average deviation is reduced to 0.7 p.p.m. and the mean to 11.2 p.p.m. On this basis the value 14.0 p.p.m. can be shown to exceed the probable deviation, and thus its elimination is justified.

The two extreme deviations, 1.5 and 1.3 p.p.m. in the timothy samples, contributed materially to the average deviation of 0.8 p.p.m. If the value 4.9 is eliminated the mean becomes 6.5 p.p.m. but the average deviation drops to 0.4 p.p.m. Elimination of this value is justified upon the basis of the probable deviation.

As noted above, W. T. Mathis was sent the three collaborative samples for spectrographic analysis. Calculated to the dry basis, his copper values are as follows in p.p.m.; alfalfa 9.2, 9.8, mean 9.6; timothy 3.9, 4.6, mean 4.2; buckwheat 3.1, 3.1, mean 3.1. These values are significantly lower than those obtained by colorimetric analysis. In the 1951 collaborative study, using the same lots of materials, the following mean values were obtained (two analysts reporting): alfalfa 10.2 p.p.m. and timothy 5.9

TABLE 1.—*Results of collaborative analyses for cobalt and copper in plant tissue. Moisture-free basis*

COLLABORATOR	SAMPLE	COBALT—P.P.M.				COPPER—P.P.M.			
		A	B	C	AV.	A	B	C	AV.
1	Alfalfa	—	—	—	0.18	—	—	—	10.6
2		0.14	0.15	—	.14	11.0	10.5	—	10.7
3		.12	.12	.13	.12	12.2	11.9	11.5	11.9
4		.13	.13	.15	.14	10.7	10.2	10.3	10.4
5		.14	.13	—	.13	12.5	12.5	—	12.5
6		—	—	—	.14	—	—	—	11.5
7		.15	.15	.13	.14	11.2	11.6	10.6	11.1
8		—	—	—	.16	—	—	—	14.0*
10		.12	.13	—	.12	12.5	11.5	—	12.0
11		—	—	—	.10	—	—	—	10.2
Means					.14				11.5
1	Timothy	—	—	—	0.06	—	—	—	6.2
2		.03	.04	—	.03	7.0	7.0	—	7.0
3		.07	.07	.05	.06	6.6	6.4	5.0	6.0
4		.03	.03	.06	.04	4.1	5.6	5.1	4.9*
5		.06	.05	—	.05	6.8	6.8	—	6.8
6		—	—	—	.04	—	—	—	6.3
7		.07	.09	.08	.08	6.1	5.9	6.2	6.1
8		—	—	—	.09*	—	—	—	7.7
10		.03	.03	—	.03	6.5	6.5	—	6.5
11		—	—	—	.04	—	—	—	5.7
Means					.05				6.4
1	Buckwheat flour	—	—	—	0.03	—	—	—	3.7
2		.02	.03	—	.02	3.7	3.7	—	3.7
3		.05	.05	.05	.05	4.0	3.7	3.2	3.6
4		.04	.03	.04	.04	2.8	3.6	3.1	3.2
5		.04	.04	—	.04	4.5	4.7	—	4.6
6		—	—	—	.02	—	—	—	4.1
7		.10	.11	.10	.10*	4.6	4.9	4.6	4.7
8		—	—	—	.09*	—	—	—	4.2
10		.03	.03	—	.03	3.8	3.7	—	3.7
11		—	—	—	.05	—	—	—	3.6
Means					.05				3.9

* These values may be eliminated on the probability they are not representative.

p.p.m. All of these data are of the same order of magnitude, and no evidence is at hand that would permit the rejection of results from either the spectrographic or colorimetric analysis in favor of the other.

COMMENTS OF THE COLLABORATORS

Dr. R. B. Carson (for Dr. Johnston).—1. We have found that the sensitivity of this modification of the nitroso-R-salt method is only about one-third of that for the *o*-nitroso-cresol or α-nitroso-β-naphthol methods. The cobalt complex appears to be attacked by bromine and the reduction in color is proportional to cobalt content.

2. The color of standards prepared by the specified method is relatively stable. Approximately the same reading was obtained after one-half hour or eighteen hours.

3. The color developed in samples containing copper, zinc, and other elements in addition to cobalt appears to be too high in the first hour after development and to fall off to about the values given by other methods in about three hours.

4. With highly mineralized samples such as alfalfa where precipitation may occur

on the addition of ammonia, it would be advisable to add 10 ml. of citrate at the start and prevent precipitation rather than try to re-dissolve the precipitate after it has formed.

5. Retention of metals and slower reaction with dithizone were noted on the calcium citrate adhering to the separatory funnel and on a small amount of precipitate gathered by shaking with carbon tetrachloride. In these cases, results by the nitroso-R-salt method were low and the residues from the dithizone extraction were shown to contain the missing cobalt when treated by the α-nitroso-β-naphthol method.

Dr. W. R Flach.—We had no difficulty with the A.O.A.C. method which we followed in detail, and we prefer this method to the nitroso-cresol method of last year. Although we did follow the dry ashing procedure with platinum dishes as outlined, we prefer the wet digestion process as it is considerably less time-consuming.

Mr. C. Tyson Smith.—I consider the cobalt results on the timothy and buckwheat to be little more than approximations, as the sensitivity of even the spectrophotometer is not too reliable at these extreme dilutions. In order to obtain greater reliability, the amount of sample ashed should be greatly increased and should be not less than 50 g.

The method of dissolving the ash is more likely to introduce errors than to eliminate them. In particular, the use of hydrofluoric acid is questioned. This acid would break down any silicates present. It is well known that some soils run as high as 50 p.p.m. cobalt, and that dust from soil adheres to hay, etc., despite the most careful washing, and may readily constitute 2 to 3 per cent of the ash, or even more. It is obvious that in such a case more cobalt would be introduced into the analysis from the adhering soil than from the plant material, which alone is available to animals.

It is hoped that if this method is adopted, a caution will be inserted in the method for copper stating that the amount of cobalt and nickel must not exceed one or two parts per million, as the carbamate method without isolation of copper actually measures both these other two elements as well.

SUMMARY AND CONCLUSIONS

Ten collaborators determined cobalt and copper in three samples of plant material using methods submitted by the Associate Referee. One collaborator reported spectrographic determinations of copper and one reported cobalt results using the α-nitroso-β-naphthol method.

The average deviations from the adjusted means of the cobalt determinations were 0.01 p.p.m. with a maximum deviation of 0.04 p.p.m. in the case of alfalfa and 0.02 p.p.m. in the two materials having a low cobalt content.

The average deviations from the adjusted means and the maximum deviations of the copper determinations were respectively: alfalfa 0.7 and 1.3 p.p.m., timothy 0.4 and 0.7 p.p.m., and buckwheat flour 0.4 and 0.8 p.p.m.

The results reported by the collaborators show that a good degree of precision is obtainable by both the cobalt and copper methods used in this study and that the results obtained are comparable to those found by other methods of analysis. In particular, the results obtained in 1951 using the nitroso-cresol method are in agreement with those obtained by

the nitroso-R-salt method. Most collaborators favor the latter method but certain advantages of the nitroso-cresol method, such as its greater sensitivity, render its use in research work important.

RECOMMENDATIONS

It is recommended*—

1. That the nitroso-R-salt method used in the 1952 collaborative study of the determination of cobalt in plants be adopted, first action.
2. That the nitroso-cresol method used in the 1949 and 1951 collaborative studies of the determination of cobalt in plants be adopted as an alternative method, first action.
3. That the sodium diethyldithiocarbamate method for copper in plants, studied collaboratively in 1949, 1951 and 1952, be adopted, first action.
4. That further collaborative work on these methods be postponed since their development is still in progress. As new methods or modifications appear, additional collaborative work should be undertaken.

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The contributed paper entitled "An Analytical System for the Determination of the Phosphorus Compounds in Plant Materials" appears on page 490.

No report was given on boron, carotene, or on sampling.

REPORT ON SPECTROGRAPHIC METHODS

SPECTROGRAPHIC AND FLAME PHOTOMETRIC STANDARDS

By W. T. Mathis (The Connecticut Agricultural Experiment Station, New Haven, Conn.), *Referee*

A collaborative study of the performance of chemical, spectrographic, and flame photometer methods was made last year (*This Journal*, **35**, 406 (1952)). An attempt has been made this year to account for some of the differences between laboratory calibration levels shown in that study.

* For report of Subcommittee A and action of the Association, see *This Journal*, **36**, 50 (1953).

As each laboratory used its own standards and standardization procedure, it seemed advisable first to examine this phase of the procedures used. A request for detailed information in this regard was sent to collaborators who used colorimetric or instrumental methods for any of their determinations. The replies from those who responded are reported, in substance, as follows:

LABORATORY NO. 1

Spectrographic Method.—(For K, Ca, Mg, P, Mn, Fe, Al, Na, Cu and B). Standards are composed of analytical grade chemicals in weak HCl solution. General standards consist of a series of stepped dilutions of a stock solution containing all of the pertinent elements in the relative proportions encountered in average plant material. Analysis curves prepared from this series of standards are used for all samples without correction for matrix element differences, except in the cases of calcium and phosphorus determinations. Additional curves are constructed for these two elements from sets of standards in which the influencing elements (potassium and potassium-calcium, respectively) are held constant at different levels while the element to be determined is varied within each of these matrix element levels. In practice, calcium and phosphorus percentages are read from curves appropriate for the matrix element composition of the particular sample. Samples are ashed and dissolved in dilute HCl. From this point the treatment of samples and standards is the same. Cobalt is added in all cases as internal standard.

Two of the general standards and twelve samples are placed on each film. Sample results are based upon the average of exposures on duplicate films, while reference points are based upon average readings for each of the two standards from the last eight or ten films run. The more or less permanent analysis curves used are not altered unless these average standard readings become seriously out of line.

LABORATORY NO. 3

Flame Photometer Methods.—(For K, Ca and Mg). Two types of standards are used. (A) *Synthetic,* which is composed of C. P. chemicals previously analyzed chemically to determine content of desired element. Each standard contains, besides the variable element, one half the maximum amount of calcium, magnesium, or potassium normally found in plant ash solutions. Sodium content is 10 p.p.m. unless sodium is the variable. The standards are 0.15 N in respect to HCl. (B) *Natural,* which is dried leaf material. This standard and two repetitions of the previous day's run are included in each day's run of 36 samples. This procedure serves as a check of both photometric calibration and over-all technique, as the checks represent complete analysis, i.e., ashing, dissolving, analyzing, etc. Total difference of 0.05 per cent, or 5 per cent of the total amount present is considered passable. Actually the differences are usually much less.

Colorimetric Methods.—(For P and Mn). C. P. chemicals are used as standards. Each new lot of KH_2PO_4 is analyzed by another method for phosphorus, after which the phosphorus standards are prepared by dissolving the proper amount of salt. The manganese standard is prepared by properly diluting a concentrated $KMnO_4$ solution which has been standardized with oxalate. After some trials it was concluded that it was not necessary to carry the standard through the entire process.

LABORATORY NO. 4

All standards are prepared from C. P. reagent grade chemicals and blanks are established for the solvents used.

Flame Photometer Methods.—(For K, Ca, Mg and Na). The composition of the

standards is as follows: (All solutions are 0.3 *N* in HNO_3. Salts used are KCl, $CaCl_2 \cdot 2H_2O$, $Mg(NO_3)_2 \cdot 6H_2O$, and NaCl.)

TABLE 1

ELEMENT TO BE DETERMINED	RANGE (p.p.m.)	STANDARD SOLUTIONS (p.p.m.)			
		K	Ca	Mg	Na
Potassium—7 standards	0–300	—	150	35	10
Calcium—7 standards	0–300	150	—	35	10
Magnesium—7 standards	0–70	150	150	—	10
Sodium—6 standards	0–100	150	150	35	—

Colorimetric Methods.—(For B, Fe, Mn and P). Appropriate aliquots of the following standard solutions are carried through color development and subsequent treatments: *Boron.* Standards, 0–7 mmg B per liter from H_3BO_3. Corning #728 boron-free glassware is used for storage of all reagents. Soft glass colorimetric and centrifuge tubes are used in determinations. Ashing is done in porcelain crucibles. *Iron.* Standard, 0.0001 g Fe per ml from ferrous ammonium sulfate. *Manganese.* Prepare standard solution containing 0.005 mg Mn/ml by adding 4.56 ml of 0.1 *N* $KMnO_4$ to 50 ml H_2O containing 0.2 ml H_2SO_4. Heat to boiling and add slight excess of Na_2SO_3 to reduce $KMnO_4$. Boil off excess SO_2 and dilute to 1 liter. *Phosphorus.* Standard 0.016 mg P/ml from KH_2PO_4.

LABORATORY NO. 5

Colorimetric Method.—(Phosphorus.) Known amounts of a pure phosphorus salt and standards are subjected to same treatment as samples.

LABORATORY NO. 6

Flame Photometer Methods.—(For K and Na). Standards are prepared by diluting stock solutions of KCl or NaCl, respectively, which contain no other salts except LiCl as internal standard. The standards are not carried through any of the preliminary treatments given the samples; only the evaluation procedure is common to both. No effort is made to synthesize standards which approximate the composition of the samples.

Colorimetric Methods.—(For Mn, Cu, Co, Pb, Zn and Mo). Blanks on reagents are used as the basis of comparison in each case. Standards, with the exception of Mo, are not subjected to ashing, digestion, and other preliminary treatments given the samples, but are introduced at the point of color development. Mo standards are carried through the entire sample procedure.

LABORATORY NO. 8

Standards are prepared from the best grade of analytical chemicals and are checked for contamination by spectrographic means. An attempt is made to use standards with a composition approximately similar to that of the unknowns, particularly with regard to Ca. Preliminary sample treatment consists of ashing and dissolving in dilute HCl.

Flame Methods.—(For K and Na). Lithium, as internal standard, and also a small amount of isopropyl alcohol are added to both standard and sample solutions.

Spectrographic Method.—(For Mg, Mn, Fe and Cu). Sample ash solutions and standard solutions are evaporated to dryness and the salt residues are dissolved in a buffer and internal standard solution.

LABORATORY NO. 10

Terms used: (1) *Zero standard*—a blank containing the same concentration of reagents as that used in the standards. (2) *Blank*—a blank carried through the entire procedure, starting with the ashing process but containing no sample material.

Flame Methods.—(For K and Na). Sets of three tung leaf samples covering the ranges of these elements are used as standards. Values of the standards are determined by chemical analysis.(No corrections were made for differences in mineral composition in the alfalfa samples in the A.O.A.C. study.)

Colorimetric Methods.—(For Mg, B, Zn, Cu, Mn, Fe and P). All curves are constructed from synthetic standards which are used in the respective procedures at the time of color development. *Magnesium and Boron.* A blank, zero standard, and multiple other standards are run with each set of samples. *Zinc and Copper.* Same, but as additional control a standard tung leaf sample is run about every fourth set. *Manganese, Iron, and Phosphorus.* A blank and zero standard are run with every set. Standards are run periodically to check curves.

LABORATORY NO. 12

Spectrographic Method.—(For Ca, Mg, P, Mn, Fe, Al, Na, Cu, and B.) Preliminary sample treatment consists of ashing and dissolving the ash in dilute HCl. Standards are synthetic and enter the procedure at this point. The series of standards used on each plate covers the anticipated ranges of elements in the particular set of samples. It is important that the standards contain the major constituents in approximately the same relative proportions as do the samples. Subsequent treatment is exactly the same for standards and samples. An analysis curve is plotted for each element from the known data on each plate, and these curves are used for evaluation of the samples on this plate only. The process is replicated three or four times to obtain average values for each sample.

DISCUSSION

A study of this information fails to reveal any obvious explanations for calibration differences between laboratories on the basis of standards and standardization procedures used. As a matter of fact it is interesting to note that, within the flame photometer group for potassium, Laboratory No. 8, with a calibration level of −0.37 per cent, took the precaution of checking the purity of chemicals used in the standards and of simulating the composition of the samples with respect to matrix elements, while Laboratory No. 6, with a calibration level in exact agreement with the group average, disregarded matrix element variation in the samples and added none of these elements to the standards. Lithium was added in both cases as internal standard.

Laboratory No. 10, with a calibration level of +.33 per cent, used natural (leaf) standards, which should represent the ultimate in procedure if the standard values can be correctly established. A comparison of the data for the other determinations with the standardization procedures used makes an equally confusing picture in this regard.

The very wide difference in copper level reported in the study for Laboratory No. 6 was subsequently found to be due to use of the wrong color filter. A later report from this laboratory showed copper values well in line with the average for the group. The wide phosphorus level for

Laboratory No. 8 is unexplained, but might possibly be due to some such cause. Laboratory precision in both of the latter cases was good and these errors could have undoubtedly been avoided if a reference sample had been included in the particular sample runs.

A.O.A.C. and other studies have shown that agricultural analyses are presently being run by spectrographic and flame photometer methods using various makes, types, and combinations of equipment. Procedures used necessarily conform to the requirements of particular equipment. In a majority of cases the results are entirely satisfactory when compared to those obtained by chemical methods. The indications are that most analysts are familiar with sound principles of standardization and follow them reasonably well. In view of this situation and what has been learned about it, it would seem that A.O.A.C. procedures for spectrographic and flame photometer analysis of agricultural materials might be set up in such a way as to permit the continued use of equipment assemblies and related procedures which are presently producing satisfactory results, and at the same time to cover very thoroughly all the basic principles involved as a guide to newcomers in the field.

The Referee has outlined information on general procedure that might be pertinent to a section on spectrographic and flame analysis, covering: (1) Specific applications, (which will be referred to the appropriate sections for preparation details, specific requirements, etc.). (2) Equipment. (3) Preliminary instrumentation. (4) Preparation of standards. (5) Evaluation of precision of technique. (6) Reference procedure. This draft will be submitted to the collaborating laboratories for criticism and suggestions. When in satisfactory form the procedure will be submitted to the A.O.A.C. for consideration with regard to inclusion in the *Official Methods of Analysis.*

ANNOUNCEMENTS

Committee Appointments

COMMITTEE TO CONFER WITH AMERICAN PUBLIC HEALTH ASSOCIATION ON STANDARD METHODS OF MILK ANALYSIS:

F. L. Mickle, Bureau of Laboratories, State Department of Health, Hartford, Conn.

COMMITTEE TO CONFER WITH AMERICAN SOCIETY FOR TESTING MATERIALS ON SOIL CONDITIONERS:

C. S. Slater, Bureau of Plant Industry, Soils, and Agricultural Engineering, Beltsville, Md.

S. J. Toth, Rutgers University, New Brunswick, N. J.

COMMITTEE ON SPECTROPHOTOMETRIC NOMENCLATURE:

B. H. Brice, Eastern Regional Research Laboratory, Philadelphia, Pa.

Referee Appointments

FERTILIZERS:

S. J. Toth, Rutgers University, New Brunswick, N. J., has been appointed Associate Referee on Soil Conditioners (performance).

PLANTS:

W. T. Mathis, Connecticut Agricultural Experiment Station, New Haven 4, Conn., has been appointed Associate Referee on Potassium.

NUTRITIONAL ADJUNCTS:

Lawrence Rosner, Laboratory of Vitamin Technology, 7737 S. Chicago Ave., Chicago, Ill., has been appointed Associate Referee on Riboflavin Concentrates.

VEGETABLE DRUGS AND THEIR DERIVATIVES:

Rupert Hyatt, Food and Drug Administration, Cincinnati 2, Ohio, has been appointed Associate Referee on Aminophyllin and Phenobarbital.

SYNTHETIC DRUGS:

Theodore E. Byers, Food and Drug Administration, Cincinnati 2, Ohio, has been appointed Associate Referee on Aspirin and Phenobarbital.

METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS:

Felix Sabatino, Food and Drug Administration, Washington 25, D. C., has been appointed Associate Referee on Aldrin.

ALCOHOLIC BEVERAGES:

W. C. Geagley, Bureau of Chemical Laboratories, Michigan Department of Agriculture, Lansing, Mich., has been appointed Associate Referee on Tartrates.

CEREAL FOODS:

Frank Collins, Food and Drug Administration, Cincinnati 2, Ohio, has been appointed Associate Referee on Bromates in Flour.

ERRATA FOR FEBRUARY, 1953, JOURNAL

In the article "Analysis of Lemon Oils" by J. W. Sale, *et al.*, **36**, 112 (1953), Table 1, page 114, "INV 30 507 H" in column headed "Base-Line Absorption (LINE CD)" change 2.29 to 0.29.

CONTRIBUTED PAPERS

DETERMINATION OF NITROFURAZONE IN FEEDS*

By Victor R. Ells,† Edward S. McKay,‡ and Henry E. Paul†

The determination of nitrofurazone, 5-nitro-2-furaldehyde semicarbazone (1), in feeds at the concentration of 0.0055 per cent usually employed in the prophylaxis of coccidiosis in poultry (2, 3), presents something of a problem. This is due to its relative insolubility in suitable solvents, its low concentration, and the considerably larger proportion of interfering pigments in the feed. However, the following method, which overcomes these difficulties reasonably satisfactorily, has been worked out for this assay. If a control feed is available (sample from feed batch before addition of nitrofurazone) and it is desired, it can be carried along simultaneously with the sample and used as a blank at the completion of the extraction step; this eliminates the reduction step of the procedure. Concentrates containing around 1 per cent of nitrofurazone and designed for mixing with feed may be assayed by the much simpler procedure outlined near the end of this paper.

METHOD

APPARATUS AND CHEMICALS

Mill.—(Cutting type, such as Wiley Intermediate).

Coarse fritted disc funnels.—15 or 30 ml Büchner Type (as Corning No. 36060, Pyrex).

Solvents.—Skellysolves C and B (Skelly Oil Co.), carbon tetrachloride, absolute ethyl alcohol, 95% ethyl alcohol, acetyl dimethylamine or dimethylformamide.

Sodium hydrosulfite.—($Na_2S_2O_4$).

Nitrofurazone.—Twice recrystallized from 50% ethyl alcohol in water.

Beckman spectrophotometer.—Model DU or equivalent instrument providing a 50 Å or narrower passband around 3700 Å.

PROCEDURE

Samples with 0.0055% nitrofurazone.—The feed samples are ground as finely as possible (passage through a 30 mesh screen was found to be adequate) consistent with a reasonable time of grinding. The mill is preferably electrically grounded to minimize static charges which might cause sepn of part of the nitrofurazone. Pledgets of clean cotton (¼″–⅜″ thick) are tamped down in the filter funnels, and while applying gentle suction, 9.1 g of the ground feed samples are placed in the respective funnels. After tamping lightly and uniformly with the same care exercised in packing chromatographic columns, solvents heated to 50–60°C. on a hot plate are passed through in the following sequence over a period of not less than 20 minutes: (1) 60 ml Skellysolve C, (2) 25 ml of a 1:1 mixture of Skellysolve C and carbon tetrachloride, (3) 50 ml of carbon tetrachloride, (4) 25 ml of the 1:1 mixture, and (5) 25 ml of Skellysolve B. This procedure removes the greater part of the interfering

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* Presented at the annual meeting of the Association of Official Agricultural Chemists, September 29–October 1, 1952, Washington, D. C.

† (Eaton Laboratories, Inc., Norwich, N. Y.)

‡ (Feed and Fertilizer Control Laboratory, Ohio State Department of Agriculture, 87 North Fourth St., Columbus 15, Ohio.)

pigments, fats, etc., without extracting significant quantities of nitrofurazone. If the last portions of solvent in (1), (3), and (5) are not practically colorless, increase vol. of solvent used; temperatures can also be increased somewhat (except with Skellysolve B). This extn procedure should not be hurried; the min. time required for extn of interfering materials will vary somewhat with the type of feed and the exact physical state after grinding. If control feeds are being used, care should be taken that they are treated and extracted in *exactly the same manner* as the corresponding medicated feeds.

Air is then sucked rapidly through the filters until the samples are dry, as indicated by the returning to room temp. of the funnels (to prevent turbidities and incomplete extn of nitrofurazone due to coating with previous solvent). The dried samples are extracted slowly with absolute ethyl alcohol at 50–60°C.; 100 ml of extract is collected at a uniform rate over a period of 30 minutes. In this step, the extracts should be protected from strong light (use red or amber glassware, or dim non-fluorescent lights). Where control feed samples are used, they are taken as blanks and the medicated samples are read against them at 3650 Å to determine the absorbance (optical density).

9.1 g of feed of nitrofurazone, concn 0.0055 per cent, contains 0.5 mg of nitrofurazone. $E_{1cm}^{1\%}$ of nitrofurazone in absolute ethanol (3650 Å) is 870. Thus:

$$\frac{\text{Absorbance} \times 10^5}{870 \times 0.5} = \text{Absorbance} \times 230 = \text{Per cent of labeled concentration of nitrofurazone, or:}$$

$$\frac{\text{Absorbance}}{0.435} \times 0.0055 = \text{Absorbance} \times 0.01264 = \text{Per cent nitrofurazone in feed.}$$

Table 1 gives the results of some analyses in which control feeds were used.

Where control feed samples are not used, or when a more accurate analysis is desired, the medicated feed extracts are treated by a procedure which obviates the necessity of identical treatment of both medicated and control samples, but which is more time consuming. It consists of the reduction of the nitrofurazone in the ethanol extracts; the reduced extract then serves as a control. Fifty ml or other desired aliquot of the

TABLE 1.—*Nitrofurazone analysis using control feed blanks (0.0055% added)*

ABSORBANCE (SAMPLE VS. CONTROL)	NITROFURAZONE	
	RECOVERY	FOUND
	per cent	*per cent*
0.466	107.0	0.0059
.450	103.4	.0057
.425	97.8	.0054
.410	94.3	.0052
.438	101.0	.0055
.444	102.0	.0056
.422	97.0	.0053
.415	95.5	.0052
.432	99.5	.0055
.418	96.5	.0053

absolute ethanol extracts are diluted 1:1 with distilled water. A solution of pure nitrofurazone of the same concentration as the amount expected in the feed sample extracts (0.25 mg in 100 ml) is also prepared in 50 per cent ethanol. This latter solution is used as the reagent reaction blank; its absorbance is zero with most lots of reducing agent, but a small correction is found with some lots.

To 25 ml or other convenient aliquot of the diluted extract (now in 50% ethanol), and reagent blank solution, are added 25 mg (or 1 mg/ml) of fresh dry sodium hydrosulfite. The solutions are allowed to stand 10–15 minutes with occasional agitation. The solutions will probably be cloudy and cannot be readily cleared by centrifugation; however, filtration through a highly retentive paper (as S & S No. 576, or Whatman No. 5 or No. 42) will clarify them. The original unreduced and reduced solutions, as well as the reagent blank, are then read at 3700–3725 Å. The absorbance of the reduced solution is corrected if necessary (reagent blank reading). The difference between this value and the reading of the original unreduced solution gives the absorbance of the nitrofurazone present if the background absorption is unaffected by the hydrosulfite. This has been the case with feeds so far investigated. The value should be 0.200 for 0.0055% nitrofurazone present and recovered, as $E_{1cm}^{1\%} = 800$ in the 1:1 ethanol water mixture.

$$\text{(Differences in Absorbancies)} \times 500 = \text{Per cent of labeled concentration of nitrofurazone;}$$

or

$$\frac{\text{(Difference in Absorbancies)}}{0.200} \times 0.0055 = \text{Per cent nitrofurazone in feed.}$$

Since some feeds may contain added riboflavin and since the pre-extraction may remove only a portion of this substance, the sodium hydrosulfite reduction of riboflavin and mixtures of nitrofurazone and riboflavin in 50 per cent ethanol was investigated. Riboflavin is reduced quantitatively in a matter of seconds to leucoriboflavin (4, 5), whose absorption at 3700–3725 Å is only a few per cent less than that of the parent riboflavin. The reaction is rapidly reversible on exposure to air. Since the reduction of nitrofurazone is much slower, and is irreversible, the leucoflavin is quantitatively reconverted to the flavin when the reduced solution is read (usually 10–15 minutes or longer after sodium hydrosulfite addition) for the background pigment absorption, and hence no appreciable interference is experienced.

Table 2 gives data on feeds to which known amounts of nitrofurazone have been added; using the reduction technique, recovery is 95 per cent or better in practically all cases. Table 3 gives some representative data on commercial feeds containing nitrofurazone. These were obtained with the Beckman Model DU spectrophotometer and a slit width of 0.32 mm. The temperature of the solutions was 25° C.

Procedure for concentrates.—Concentrates, prepared for mixing with feed, and generally containing 1.1% nitrofurazone, may be analyzed directly by extraction with acetyl dimethylamine or dimethylformamide (Eastman Nos. 4972 and 5870).

TABLE 2.—*Results by hydrosulfite reduction with known added amounts of nitrofurazone*

NITROFURAZONE ADDED	SAMPLE NO.	ABSORBANCE			NITROFURAZONE FOUND
		A_1 (UNREDUCED)	A_2 (REDUCED)	A_1–A_2	
per cent					*per cent*
0.0045	1	0.319	0.152	0.167	0.0046
	2	.314	.150	.164	.0045
	3	.321	.157	.164	.0045
.0050	1	.297	.124	.173	.0048
	2	.297	.116	.181	.0050
	3	.314	.135	.179	.0050
.0055	1	.332	.132	.200	.0055
	2	.319	.122	.197	.0054
	3	.337	.134	.203	.0056
.0060	1	.340	.125	.215	.0059
	2	.362	.147	.215	.0059
	3	.367	.149	.218	.0060
.0065	1	.369	.131	.238	.0065
	2	.387	.147	.240	.0066
	3	.372	.134	.238	.0065

TABLE 3.—*Results on commercial feeds (all 0.0055 per cent labeled concentration)*

SAMPLE NUMBER	ABSORBANCE			NITROFURAZONE FOUND
	A_1 (UNREDUCED)	A_2 (REDUCED)	A_1–A_2	
				per cent
1	0.310	0.143	0.167	0.0046
2	.340	.161	.179	.0049
3	.382	.192	.190	.0052
4	.469	.276	.193	.0053
5	.403	.206	.197	.0054
6	.380	.179	.201	.0055
7	.441	.237	.204	.0056
8	.357	.149	.208	.0057
9	.409	.194	.215	.0059
10	.377	.156	.221	.0061
11	.406	.176	.230	.0063
12	.412	.164	.248	.0068
Average				.0056*

* The average value for 32 samples of various types of feed, all falling within the range of the above and including them, was 0.00557).

It was not found necessary to grind the concentrates provided that they were carefully sampled. Eleven ml of solvent per gram of sample is added and the flasks are shaken for 10–15 minutes. The extracts are decanted and centrifuged for clarification, and 100-fold dilutions in a 50% ethanol in water solution are made. At 3700–3725 Å, the absorbance is 0.80 for 100% of labeled nitrofurazone content, as $E^{1\%}_{1cm} = 800$ in this solvent. The analysis on concentrates can also be carried out using 95% ethanol as extractant in the proportion of 44 ml per gram of sample. The flasks are shaken for one hour, and 25-fold dilutions are made in 50% ethanol in water. The dilutions may also be made in 95% ethanol instead of 50% in both of the above extraction procedures. In this case, the diluted solutions are read at 3660 Å, with $E = ^{1\%}_{1cm} 840$.

Analyses were carried out on a sample of Nefco (A. J. White, Ltd., London, England) which contains 1.1% nitrofurazone. Observed absorbances (in 50% ethanol in water) were 0.81 and 0.81 by the first procedure and 0.80 and 0.80 by the second (101.3 and 100% of the label declaration, respectively).

It is, of course, possible that in the future, complex concentrates might be marketed containing sufficient quantities of interfering additives to require modification of this procedure. Sodium hydrosulfite reduction of the solutions (in 50% ethanol) as outlined above would eliminate effects due to riboflavin and probably those due to at least some of other possible additives. Others, e. g., nitrophenide, would be removed during a pre-extraction.

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REACTION OF CALCIUM CARBONATE WITH SOILS AND DETERMINATION OF THEIR CALCIUM SORPTION CAPACITIES

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Scientific investigation of the proper utilization of liming materials on acidic soils had its beginning in the early agricultural research. Although liming exerts various physical, chemical, and biological effects upon the soil, its principal benefit is the neutralization of soil acidity. Crop growth in the field and in the greenhouse is employed as a convincing test for the kind and rate of liming best suited for a given soil area. However, because of the cost and other difficulties connected with such tests, soil scientists have devised simple chemical procedures for testing the soil's need for

liming. Of such tests the simplest now in use is the *p*H determination by means of the glass electrode. To a large extent, the *p*H value serves as a reliable criterion of the soil's calcium supply, but as a means of predicting the quantity needed to raise the soil to a desired *p*H, it cannot be relied upon without complicated computations and the aid of additional data (8, 9, 35, 40) or the employment of intuitive judgment of persons (11, 12, 36) familiar with the practical requirements of the area. It is for these reasons that in addition to the *p*H determination there also is a need for chemical methods for the quantitative determination of "lime requirements" of soils.

The objective of the present investigation is to determine the rates of reaction of soils and soil clays with finely divided calcium carbonate under different moisture and temperature conditions and to establish reaction capacities of soils under feasible laboratory conditions. Chemical methods specifically referred to or implied as lime requirement methods range from the mild treatment for hydrogen replacement with neutral salt solutions (16, 23, 38) to the more drastic treatment with hydroxides and other solutions at *p*H above 8 (7, 18, 32, 33, 48). In this investigation it is hoped to obtain the maximal reaction between soils and $CaCO_3$, and this should indicate maximal lime requirement. Furthermore, it is possible that when the reaction capacity of a soil has been obtained, the lime requirement may then be expressed as a degree of the calcium sorption capacity. At any rate, since calcium carbonate supplied as ground limestone is the only means of restoring calcium to the soil, it is helpful to acquire a knowledge of the soil's potential capacity to react with this material.

Because of the common objectives of many reported investigations, it will be necessary to give a critical review of pertinent publications in order to permit comparison with the procedures herein described.

REVIEW OF PREVIOUS INVESTIGATIONS

A. METHODS BASED ON PURELY CHEMICAL REACTIONS

The earliest methods for determining soil acidity were based upon the reaction of the soil with an excess of $CaCO_3$. The oldest of such methods is the Tacke procedure (47) which consisted of suspending an excess of $CaCO_3$ with the soil and aspirating the evolved CO_2 three hours at room temperature. Independent of the Tacke procedure, Wheeler, Hartwell, and Sargent (51) have investigated the possibility of the reaction of soil with a suspension of $CaCO_3$ at boiling temperature with utilization of the evolved CO_2 as a measure of lime requirement. They found, however, that there was apparently no reasonable time limit within which the elimination of carbon dioxide would be ended and observed: "It is a question of too great scientific and practical interest to be laid aside at this point, and it is hoped that opportunity for further pursuit of the question will be afforded in the near future."

The Veitch method (49) was introduced in 1902 as a modification of the

Tacke procedure. It consisted essentially of a serial titration of the soil with $Ca(OH)_2$ followed by evaporation of a 50 ml portion of the filtered water extract to about 10 ml and testing for alkalinity with phenolphthalein or, as later recommended (50), red litmus paper. The first quantity of lime that induced an alkaline reaction was taken as the lime requirement. The Veitch procedure was considered the most reliable in the first two decades of this century. Because of poor reproducibility (15, 28, 46) and the laborious nature of the determination it was abandoned. Extensive investigations by MacIntire (25, 27, 28) of the reaction of soils with $CaCO_3$ and $MgCO_3$, both in the field and laboratory, led him to conclude that soils have capacities to decompose $CaCO_3$ under field conditions greatly beyond the Veitch lime requirement indication. MacIntire drew a distinction between the "immediate" and "continued" lime requirements, and devised a procedure (26) for determination of the "immediate lime requirement." His procedure consisted of evaporating calcium bicarbonate-soil suspensions to a thin paste on a steam bath and determining the soil-$CaCO_3$ reaction from analysis of the residual $CaCO_3$. In the study of the method it was reported that precipitated $CaCO_3$ from different sources failed to give concordant results because of differences in particle size of the samples. The use of the bicarbonate solution was recommended as the only means of assuring uniform results, independent of the carbonate source.

First collaborative study of this method was reported at the 1915 meeting of the A.O.A.C. by Ames (1). The conclusion drawn was that "the amount of soil used and the conditions of evaporation in the MacIntire procedure affect the results obtained to an extent which prevents this from being a practical method" (1, p. 136). Investigation by Howard (19) of this method (26), and of Hutchinson and MacLennan (20), has shown that the results by both methods were greatly affected by variation in sample size and volume of calcium bicarbonate solution. Keeping the bicarbonate volume constant, and adding purified precipitated $CaCO_3$ to augment the supply of $CaCO_3$, failed to give satisfactory results. Ames and Schollenberg (2) proposed a modification of the Wheeler, Hartwell, and Sargent procedure. It consisted of boiling a water suspension of 20 g of soil and 2 g of $CaCO_3$ under a vacuum of 70 cm of mercury for two and one-half hours and using the evolved CO_2 as a measure of the lime requirement of the soil. This procedure was investigated by Knight (24), who found the results higher than those obtained by titrating with $Ca(OH)_2$ to *p*H 7 by the hydrogen electrode.

A more extensive collaborative study by MacIntire (29), then Associate Referee on lime requirements of soils, was reported at the 1917 meeting of the A.O.A.C. In this study ten soils were selected from plots of the Pennsylvania, Rhode Island, Maryland, and Cornell Experiment Stations; five of these reacted alkaline and the other five acidic by the Veitch test.

Among the methods studied were those of Tacke, MacIntire, and, for the first time, the potentiometric titration with $Ca(OH)_2$ using the hydrogen electrode (42). The novel feature of this study was to apply consistency tests for the various procedures through the application of precipitated $CaCO_3$ to the soils according to the requirement indicated by each procedure, and again to test the treated soils after various periods of incubation for lime requirement. Using this criterion, the potentiometric titration with $Ca(OH)_2$ to *p*H 7 after a contact period of three days gave the most consistent results and after sixteen days of moist contact with $CaCO_3$ the soils attained *p*H values very close to 7. The Tacke and the MacIntire methods, on the other hand, indicated additional carbonate decompositions of 36 and 21 per cent, respectively, on soils that were kept four weeks in moist contact with precipitated $CaCO_3$, added according to the need indicated by these procedures. This is remarkable since the indications of the average lime requirement by the potentiometric titration, the Tacke, and the MacIntire methods were in the ratios of 1:2:3, respectively. It should be noted, however, that by the Tacke and MacIntire methods the $CaCO_3$-treated soils still contained some residual $CaCO_3$ after four weeks of moist contact. In spite of great difference in $CaCO_3$ indications by the two methods, the *p*H values determined after two weeks of moist contact with respective $CaCO_3$ treatments were 7.0 to 7.3 from the Tacke treatment and 7.3 to 7.4 from the MacIntire treatment. It may be also of interest to note that four of the soils indicated as alkaline by the Veitch test showed *p*H values ranging from 5.6 to 6.4.

Later, MacIntire (29) declared that "without, in any sense, being derogatory to other procedures, which may be well adapted to certain conditions, it seems to be the consensus that the Jones calcium acetate method (22) fills the need of any absorption method to replace the abandoned sodium nitrate procedure." The Jones method calls for trituration of a 5.6 g soil sample with 0.5 g of pure calcium acetate in H_2O and ultimate dilution to 200 ml. This salt concentration is only about 0.025 times as concentrated as salt concentrations normally used in base exchange studies. The Jones method may be regarded as essentially an equilibrium hydrogen replacement by means of a very dilute calcium acetate solution and is vitiated only by the initial akalinity of the pure salt.

A review of lime requirement methods covering the period from 1900 to 1920 (most of which were studied under the auspices of the A.O.A.C.), does not establish which of the several possible reasons was responsible for abandonment of the calcium carbonate or bicarbonate procedures for lime requirement. These may have been because (a) the soil-$CaCO_3$ reaction appeared to be continuous and was lacking in precision, (b) the lime requirement indications proved to be excessive from the standpoint of practical agriculture (17), and (c) the procedures were time-consuming, or otherwise not adapted to laboratory routine.

The need for a dependable lime requirement method has been met through the procedure, based on replacement of exchangeable hydrogen with normal calcium acetate at *p*H 7, which was presented for adoption by the A.O.A.C. (44, 45). However, the original problem, which was to determine the potential capacity of soils to react with $CaCO_3$, was side-tracked after twenty years' effort at the start of the first World War.

B. METHODS INVOLVING PHYSICOCHEMICAL MEASUREMENTS

After the attempts to utilize the calcium bicarbonate-carbonate reactions with soils to measure lime requirement had come to an unsuccessful conclusion, a new approach in the application of the reaction between calcium bicarbonate and soils made its appearance. In 1919, Bjerrum and Gjaldbaek (5) made an epochal contribution to the study of acidic and basic properties of soils by means of titration or buffer curves, and established the relationship between partial pressure of CO_2 and *p*H values of saturated solutions of $CaCO_3$. Based upon the findings of these investigators, Jensen (21) introduced a method for titration of acidic soils with $Ca(OH)_2$ solution, followed by equilibration with the partial CO_2 pressure of the atmosphere. The *p*H values plotted against the $Ca(OH)_2$ additions form the buffer curve. The advantage of such a curve is that it gives the lime requirement of the soil at different *p*H values, including *p*H 8.4, the point where the soil is in equilibrium with the surplus of $CaCO_3$. However, this method is not well adapted for obtaining the Ca-saturation value of the soil, because as pointed out by Bradfield and Allison (7), the small excesses of bicarbonate in solution are not readily precipitated upon aeration. Those workers (7) made a thorough study of the soil-$CaCO_3$ equilibration, using conductometric and potentiometric titration curves for recognition of the Ca-saturation end point. After discussion of the relative merits of the titration procedures as against analysis of residual $CaCO_3$, they concluded that the residual carbonate analysis "is unquestionably the most accurate method" (7, p. 74). A further objection to the conductometric and potentiometric procedures is the fact that eight to ten samples are necessary. From a study of the several stages of contact before equilibration, it was concluded that with the sixteen-hour aeration the $Ca(OH)_2$-soil contact need be only one hour, but twelve-hour contact was usually allowed. After sixteen-hour contact with $Ca(OH)_2$ and carbonation, the time of aeration sufficient for correction for $Ca(HCO_3)_2$ in solution is four hours, although usually sixteen hours is allowed. Furthermore, the addition of $CaCO_3$ followed by CO_2 and sixteen-hour aeration gave results equally as good as those by the $Ca(OH)$-CO_2-aeration treatment on three soils.

In advancing their procedure as "criteria of base saturation" in soils, Bradfield and Allison pointed out that $CaCO_3$ is the most common form of alkali reserve in the soil, and that any definition of a saturated soil based on $CaCO_3$ equilibration is closely correlated with pedological processes and should have universal application. In comparing this concept

with others in regard to base saturation or exchangeable hydrogen, Bradfield observed (6) that "it has long been known that even neutral soils will adsorb enormous quantities of bases from strongly alkaline solutions." (18). "The soil chemist is usually not concerned with this adsorption." He then offered the following definition of a "base-saturated" soil. "A soil is saturated with exchangeable bases if the addition of $Ca(OH)_2$ results in the formation of free $CaCO_3$ or $Ca(HCO_3)_2$ on exposure to air." Bradfield and Allison in 1933 (7) restated the above definition in a more precise manner: "A soil saturated with bases is one which has reached equilibrium with a surplus of $CaCO_3$ at the partial pressure of CO_2 existing in the atmosphere and at a temperature of 25°C." It should be pointed out that Bradfield and Allison were aware of the difference between the quantities of exchangeable hydrogen neutralized through the equilibration with $CaCO_3$ and that which is involved in the neutralization by titration to *p*H 7 only. They declared, however, that there was " . . . no cogent reason why it should not be included in the calculation of the base absorbing capacity of soils."

A number of investigators have accepted the Bradfield concept of a base-saturated soil and used the soil-$CaCO_3$ equilibration as the basis of soil treatment, but they have not always followed with the determination of residual carbonate. Naftel (37) commented upon the need for a more suitable method for use in field and greenhouse liming and proposed the use of a series of liming increments based on the $CaCO_3$-equilibration saturation as the constant for each soil. However, he considered the $CaCO_3$-equilibration status to be the first point of maximal *p*H value in a series of $Ca(OH)_2$ additions that were followed by passages of CO_2 and air, a procedure identical to the one used by Jensen (21) and rejected by Bradfield and Allison as unreliable. Those authors expressed their belief that the determination of the residual carbonate, after equilibration, is essential to a true measure of the calcium sorption. Davis (13) used the soil-$CaCO_3$ equilibration procedure for the preparation of partially calcium-saturated soils. Also in this instance, determination of residual carbonate is not mentioned, and it is not certain that the saturation point was recognized.

Based largely upon Bradfield's concept of $CaCO_3$ equilibration at *p*H 8.4, Mehlich (31, 32, 33, 34) advocated the use of the highly buffered triethanolamine-$BaCl_2$ solution at *p*H 8.2 for exchangeable hydrogen replacement and rated that procedure as being more adaptable in routine operations than the Bradfield and Allison procedure. The adoption of the triethanolamine-$BaCl_2$ or any other complex chemical (52) as *p*H buffers, however, places the entire emphasis on the *p*H value alone, and disregards the specific effects that these chemicals may exert upon the soil complex. At any rate, reagents are being used in a manner that does not emphasize completed reactions, but are qualified through arbitrary contact period

and as to ratios of soil to solution, which obviously cannot fit all situations.

Recently, Patel and Truog (39) reintroduced the calcium bicarbonate-soil reaction as a basis for determining the lime requirement of soils. The described procedure is essentially the one proposed by MacIntire in 1915 (26) except that Patel and Truog evaporate the soil suspension to dryness instead of to a "thin paste." The new procedure also prescribes the gasometric analysis of the residual carbonate by means of a calcimeter. It is claimed that the results by means of the proposed method do not vary more than one-tenth ton per acre from the results obtained in the field. Furthermore, in greenhouse pot tests, soils limed according to the lime requirement indications of this method were brought to near neutral point and maintained that reaction for six months. Undoubtedly the featured evaporation to dryness has contributed to some additional calcium sorption; this has been observed in previous investigations (2) of the MacIntire procedure. It is difficult to see, however, how the evaporation to dryness or an additional evaporation of 25 ml of H_2O could erase the inherent handicaps of the procedure because results are greatly affected by reagent excess, soil sample size, and speed of evaporations (2, 19, 46).

A review of procedures and concepts of lime requirement, base saturation and exchangeable hydrogen, particularly those involving the use of calcium carbonates and bicarbonates, serves to establish the fact that soil investigators believe that the soil-base saturation capacity, or its exchangeable hydrogen, should be measured through the natural reaction of an acidic soil and additions of $CaCO_3$. The earlier procedures were not accepted because they appeared to give indeterminate values and also because those values appeared excessive in relation to crop requirements. The later physicochemical approach of $CaCO_3$-CO_2-soil equilibration has established the precision of such procedure, and has correlated the soil-$CaCO_3$ equilibration with natural physical and chemical transition points in soil pedology. In view of the universal use of factors by most titration procedures for lime requirement (3, 4, 10, 14, 41), it is not improbable that the indications by the $CaCO_3$ equilibration procedure may preclude the use of such factors. It is recognized (40), however, that the soil-$CaCO_3$ equilibration procedure of Bradfield and Allison is not well suited for routine determinations.

TECHNIQUES IN SOIL-CARBONATE REACTION STUDIES

In recent years the reaction between soils and calcite has been under renewed investigation at the Tennessee Agricultural Experiment Station as a part of the major study of the relative availabilities of various types and of different particle sizes of liming materials. A survey of the literature led to the conclusion that neither the rate of reaction nor the soil's ultimate capacity for calcite decompositions has been investigated in sufficient detail with respect to effect of moisture, temperature, proportion,

and fineness of materials. The techniques of soil carbonate reaction have been developed in the course of several years of study of this problem (44, 45). Each procedure has advantages peculiar to it. The greater part of the present investigation on the speed of calcite reaction was carried out at 30°C. and at 95°C.

A. STANDARD PROCEDURE

Samples of 100 g of air-dried soil of 1 mm fineness were mixed with an excess of 325-mesh calcite (usually one gram) and triturated lightly in a mortar to provide a uniform mixture which then was mixed into the remainder of the soil on a sheet of glazed paper. Each mixture was placed in a 150 ml beaker, except for highly reactive organic and montmorillonitic materials for which 250 ml beakers were found more suitable. The soil-carbonate mixtures were moistened, placed in a water-bath, and there held for the desired time at the desired temperature. The moisture lost through evaporation was restored daily, or at other intervals, by bringing the experiment to the initial wet weight. At the termination of the experimental period, the wet soils were spread on sheets of paper and air-dried in an atmosphere free of laboratory fumes. The soils were ground to pass a 0.5 mm sieve, mixed thoroughly, and bottled. Ten-gram samples were used for determinations of residual carbonate by means of the steam distillation procedure (43). The residual carbonate value is obtained as the difference between the acid titrations of the excess NaOH in the $BaCO_3$ suspension resultant of the carbonate treatment and that resultant of the untreated soil. The titration value on the untreated soil will be designated as "soil-reagent blank." By the use of the standard procedure, sufficient soil is provided for replicate carbonate analyses and also for multiple determinations of exchangeable hydrogen and *p*H values.

B. UNIT PROCEDURE AT ROOM TEMPERATURE

(This procedure makes use of a ten-gram soil sample and was employed primarily for short duration experiments.) The soil and carbonate were mixed in an agate mortar, avoiding excessive pressure that might crush rock particles. The mixture was placed in paraffined 28–32×34 mm soufflé cups. The desired amount of water was added and the cup was set in a 100 ml Berzelius beaker filled with water and placed in a water bath at constant temperature. (The use of an empty paraffined cup between the water in the beaker and the soil container prevented wetting of the outside of the experimental container. The cups were identified by means of markings applied to the upper inside surface before paraffining.) A record of the wet weight of each experiment permitted the periodic restoration of moisture loss.

At the expiration of the experimental period, the soils were air-dried, the rim of the cup was cut off, and the soil sample with the remainder of the cup was introduced into a flask for CO_2 determination. This technique eliminates the labor of grinding and mixing that is necessitated when larger charges of soil are used, and lessens experimental errors.

C. UNIT PROCEDURE AT BOILING TEMPERATURE

In this procedure a 10 g charge of soil and the requisite quantity of 325-mesh calcite was introduced directly into the 125 ml extraction flask, there wetted with 10 ml of H_2O and triturated with a stout rubber-tipped, slightly curved glass rod. The interior of the flask was washed down with a stream of about 20 ml of water, and the flask was placed over a 250 ml beaker on a hot plate maintained about half full of boiling water. When completely dry, the soil was wetted again with 30 ml of H_2O and the contents swirled. A single evaporation requires about one hour and

thirty minutes, and four such evaporations may be completed during a working day. After the last evaporation, the soil is ready for carbonate analysis in the same flask.

RESULTS

Results of Soil-Calcium Carbonate Reaction at 30°C.—Four soils and subsoils supplied with 325-mesh calcite in excess were incubated under conditions described as "standard procedure." The moisture conditions are given in Table 1, and losses were replenished Monday, Wednesday, and Friday of each week. The objective of this experiment was to obtain basic information on the progress of calcium carbonate-soil reaction as affected by the type of adsorption complex, and to establish any natural transition in the rate of reaction that would supply a dividing line between the "immediate" and "continued" lime requirement. Furthermore, the reactions at 30°C. were considered the nearest approach to natural conditions that can be performed in the laboratory, and the results obtained at that temperature would serve as tentative standards to which other results would be compared. The results on soil plus $CaCO_3$ reactions under standard conditions are given in Table 1.

Special attention is directed to the carbonate decompositions at the two, thirteen, twenty-six, and fifty-two-week periods and to an over-all appraisal of trends.

The reaction progress of the two samples of the organic-kaolinitic Hartsells soil for the respective periods were, for the 1947 sample: 12.5,

TABLE 1.—*Soil-calcium carbonate reaction progress during various periods up to one year under continually wet contact at 30°C.*

SOIL	$CaCO_3$ ADDED PER 100G	H_2O ADDED PER 100G	CARBONATE DECOMPOSITIONS—MEQ. PER 100 GRAMS (PERIODS IN WEEKS)								
			1	2	4	8	13	19	26	39	52
	meq	*grams*									
Experiments from October 1950 to October 1951											
Hartsells fine sandy loam, 1947	20	44	11.7	12.5	12.8	12.9	13.7	15.0	15.0	15.6	15.8
Cumberland clay subsoil, 1950	20	44	9.3	9.9	10.4	10.4	10.8	—	12.0	12.4	12.4
Susquehanna clay subsoil, 1950	40	60	28.9	28.1	29.8	30.0	30.4	31.1	31.4	32.0	31.2
Portsmouth muck, 1950	80	75	50.1	57.4	57.9	58.8	58.2	59.0	63.0	62.0	61.3
Repeats of Above Experiments, February 1952											
Hartsells fine sandy loam, 1947	20	—	—	—	12.8	14.5	14.7	—	—	—	—
Cumberland clay subsoil, 1951	20	—	—	—	10.4	11.1	11.4	—	—	—	—
Supplementary Experiment, May 1951 to May 1952											
Hartsells fine sandy loam, 1950	10	50	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
	20	50	—	—	18.0	19.0	19.0	—	19.7	19.4	20.0
	30	50	15.7	17.8	20.1	20.6	21.6	21.6	21.2	22.7	25.1

12.7, 15.0, and 15.8 meq.; for the 1950 sample: 17.8, 21.6, 21.2, and 25.1 meq. If we designate the two-week results as the "immediate" lime requirement, the increased reaction of the 1947 sample in the additional periods were 1.2, 2.5, and 3.3 meq., or 10, 20, and 26 per cent increases above those in the two-week period for the periods of three, six, and twelve months, respectively. The corresponding increases for the 1950 Hartsells soil were 3.8, 3.4, and 7.3 meq. or percentage increases of 21, 19, and 41 for the respective longer periods. Despite the steadily increasing carbonate decomposition between the two-week and fifty-two-week periods, there are many instances of apparent stoppage of the reaction between some of the periods. One instance is the two, four, and eight-week results and again in the nineteen and twenty-six-week results of the 1947 samples. Another instance is the thirteen, nineteen, and twenty-six-week results of the 1950 samples. These results are noted in detail to show how it is possible, under such circumstances, to obtain apparent soil-$CaCO_3$ reaction equilibrium where the reaction is carried only to the first two or three points of agreement on the time scale. On the other hand, the frequent appearance of equilibrium may be taken as an indication that the reaction beyond the two-week period was progressing at a very sluggish pace and that the occasional sudden upsurges in reaction might have been due to unrecognized disturbing effects. (An appreciable part of the periodic carbonate decompositions was due to the neutralization of the biologically-engendered acids, chiefly HNO_3. The cumulative nitrate in the Hartsells soil was equivalent to more than 1 meq.)

In the two-week period, the reaction between the kaolinitic Cumberland subsoil and the $CaCO_3$ amounted to 9.9 meq. and was followed with successive increases of 0.9, 2.3, and 2.5 meq. for three, six, and twelve-month periods, respectively. In a repetition of this experiment (middle section of Table 1) the increase for the three-month period was 1.5 instead of .9 meq. Equilibrium appears to have been attained in the twenty-six-week periods with 12.2 meq. $CaCO_3$ decomposition and without further increases in the thirty-nine-week and fifty-two-week periods.

The reaction between the montmorillonitic Susquehanna clay subsoil and added $CaCO_3$ amounted to 28 to 29 meq. $CaCO_3$ in the first two weeks, with 1.4 meq. additional in three months, and about 2 meq. additional distributed between the six- and twelve-month periods. There was a gain of 7 per cent in reaction between the two-week and one-year periods.

The reaction between the Portsmouth muck and $CaCO_3$ in the two-week period amounted to 57.4 meq. and to only 58.2 in the three-month period, and 62.0 ± 1 meq. in the six and twelve-month periods. When allowance is made for the 3 meq. of the $CaCO_3$ decomposed through the neutralization of engendered HNO_3, the reaction in the two-week period was only 2 meq. short of the maximum attained in one year.

Because of the proportionately high extent of reactions in the two-week

period, particularly in the case of the Susquehanna subsoil and Portsmouth muck, it appeared possible that, under certain conditions, the reaction of the two-week period could be expedited greatly. Attempt to speed the reaction in the two-week period through increasing moisture content proved partially successful in as much as the two-week reaction was 62.4 meq., as can be seen from the data of Table 2. Of the four materials tested, only the Portsmouth muck registered greater reactivity under supersaturated moisture content. The results of Table 2 have not altered

TABLE 2.—*Soil-calcium carbonate reaction—2 weeks at saturated moisture content at 30°C.*

SOIL	$CaCO_3$ ADDED PER 100G	H_2O CONTENT	$CaCO_3$ REACTION PER 100G
	meq.	*per cent*	*meq.*
Hartsells sandy loam, 1950	30	100	17.7
Cumberland clay subsoil, 1950	20	100	10.2
Susquehanna clay subsoil	40	130	30.0
Portsmouth muck	80	150	62.4

appreciably the conclusions drawn from the data in Table 1 as to rate of reaction at 30°C.

Results of the Soil-$CaCO_3$ Reaction at 95°C.—Because higher temperature induces greater speed of chemical reaction, it seemed desirable to establish the limits of this soil-$CaCO_3$ reaction as related to time of contact and to compare with the speed with which carbonate decomposition occurs in some soils at 30°C.

Effect of Multiple Evaporations upon Extent of Soil-$CaCO_3$ Reaction.—After the four representative soils and subsoils of Table 1 and their respective additions of $CaCO_3$ had been subjected to wetting and drying on a steam bath from one to sixteen times, the residual $CaCO_3$ was determined (Table 3). In the Hartsells soil, the progressions in carbonate decomposition were about 2 meq. for each reaction period of one, two, four, and eight evaporations; the increases between the eight, twelve, and sixteen evaporation periods were only about 0.7 meq. each. It appears that near maximal carbonate reaction can be attained in the Hartsells soil by wetting and drying four to eight times on the steam bath.

The reaction progress of the kaolinitic Cumberland clay subsoil follows the same pattern as that shown for the Hartsells soil, in that the near maximal values were attained after eight evaporations.

The montmorillonitic Susquehanna clay subsoil attained its maximal carbonate reaction value of 32.7 meq. upon eight evaporations on the steam bath.

The decomposition from the 90 meq. additions of $CaCO_3$ to the highly organic Portsmouth muck reached 66.7 meq. as the result of four evapora-

TABLE 3.—*Rate and extent of reaction of soils and subsoils with $CaCO_3$ as affected by the number of evaporations on steam bath*

SOIL TYPE	$CaCO_3$ ADDITION	$CaCO_3$ REACTIONS AFTER 30 ML H_2O EVAPORATIONS TO DRYNESS					
		1	2	4	8	12	16
	(meq.)						
Hartsells fine sandy loam, 1950	30	15.8	18.6	21.4	23.3	23.9	23.9
		16.3	18.5	21.9	23.6	23.3	23.9
		16.2	18.3	21.3	23.1	24.1	24.0
	(Av.)	16.1	18.5	21.5	23.3	23.8	23.9
Cumberland clay subsoil	20	11.0	12.4	13.2	14.0	14.3	15.2
		10.7	12.9	13.3	14.2	14.5	15.0
		11.5	12.5	12.9	14.4	14.8	15.1
	(Av.)	11.1	12.6	13.1	14.2	14.5	15.1
Susquehanna clay subsoil	40	29.0	31.2	30.6	32.9	32.5	33.3
		28.7	30.7	30.7	32.7	32.6	32.7
		28.2	30.1	31.3	32.6	32.7	32.8
	(Av.)	28.6	30.7	30.9	32.7	32.6	32.9
Portsmouth muck	90	48.6	56.1	66.1	69.1	70.4	70.8
		49.3	55.8	67.0	72.0	71.9	70.8
		50.3	58.3	67.0	73.0	74.4	73.0
	(Av.)	49.4	56.7	66.7	71.4	72.2	71.5

tions on the steam bath. A further increase of 4.7 meq. in reaction ensued when eight evaporations were imposed, whereas the twelve and sixteen evaporations caused only insignificant increases. The progressive reactions at 95°, in relation to time, are shown graphically in solid lines in Figure 1. Each of the curves representing reaction progress at 95°C. appears to be composed of two parts—one representing a rapidly decreasing rate of reaction, i.e., the part of continually changing slope; the other on which the points lie in a straight line. The division line for the four curves seems to fall on the two-day period which indicates that eight evaporations require about sixteen hours of actual time on the steam bath. The significance of this division line becomes apparent from a comparison of reaction values for the first eight evaporations with the reaction advances for an equal period immediately following. These values are 23.3 against 0.6 for the Hartsells soil; 14.2 against 0.9 for the Cumberland clay subsoil; 32.7 against .0 for the Susquehanna subsoil; and 71.4 against 1.0 for the Portsmouth muck. Indeed, the reaction gains in the second eight-evaporation period are so small that they could be disregarded in relation to the

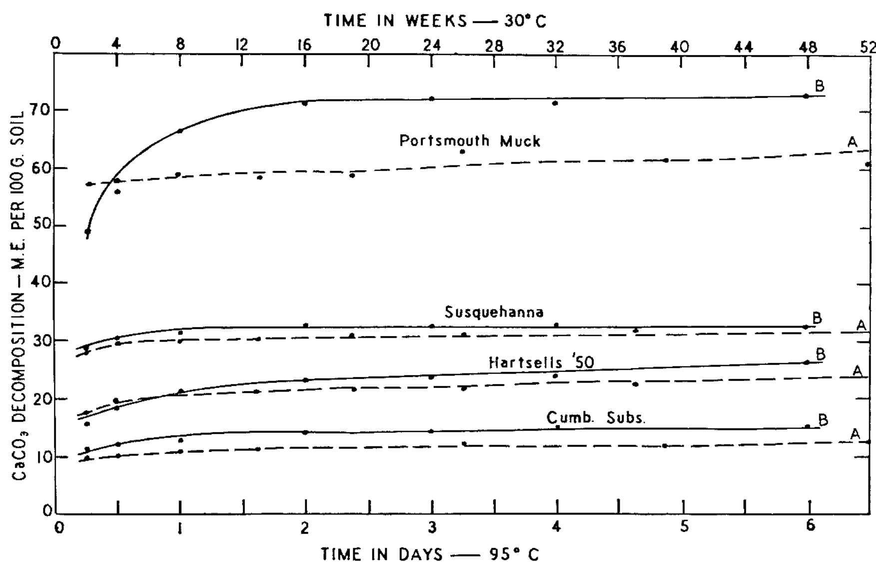

FIG. 1.—Reaction progress of soils and subsoils supplied with excesses of 325-mesh calcium carbonate (A) at 30°C. and (B) at 95°C.

total reaction. A third period of eight evaporations caused an appreciable gain in the decomposition of $CaCO_3$ in the Hartsells soil, but practically no gain in the other three soils. It is safe to conclude that a small gain in reaction continues indefinitely beyond eight evaporations, but these gains are so small that they are difficult to establish with precision (Table 6).

COMPARATIVE REACTION RATES OF FOUR SOILS AT TWO TEMPERATURES

The relative speed of reaction between several soils and added $CaCO_3$ at 30°C. and 95°C. is also shown in Figure 1. Two kinds of differences are noted: one in relation to speed and the other to ultimate values. Values agree only at the first two points on the time scale, when the plotted time scales for the 95°C. and 30°C. temperatures are in the ratio of one day to eight weeks respectively. According to these results (which hold true for the four soils) a reaction value that results from eight weeks of continued moist contact at 30° can be completed in one day at 95°. More correctly stated, a single two-hour evaporation at 95° will accomplish the same extent of soil-$CaCO_3$ reaction that would be attained in one week at 30°C. Each one-day marking on the 95° temperature scale of Figure 1 corresponds to four evaporations of 30 ml of water, each requiring about eight hours, whereas the time on the 30°C. scale represents continuous contact time.

The reaction values obtained at the temperature of 30°C. were set up

as provisional standards in judging results from different experimental conditions. By those standards, the reactions at 95° are about 2 meq. higher for the three mineral soils and subsoils, but considerably higher (10 meq.) for the Portsmouth muck. Repeated short-time experiment with the Portsmouth muck from the sample used in the 95° experiment has shown that the reaction in one week at 30° was nearly 67 meq., which indicates that the difference observed in Figure 1 was due to the lower sorption capacity of the older sample of Portsmouth muck. The prime concern in this comparison is to determine whether the higher temperature would give results that might be considered out of range of those obtained at ordinary temperature. In view of the circumstances under which the long range experiments were conducted (continually wet and without stirring), and in view of the irregularities that appeared during the reaction progress, it is not unreasonable to conclude that the results obtained at 95° are within the normal sorption capacities, and that such values would be obtained also at 30° in case the experiments were continued for longer duration or with better mixing of the reaction systems. Moreover, the long-time experiments, up to one year, not only require considerable attention, but also are subject to hazards of contamination and to interruptions because of electric current failures, etc. Then too, the long range experiments at 30° cannot be expected to give good reproducibility of results.

EFFECT OF WATER VOLUME UPON SPEED OF SOIL-$CaCO_3$ REACTION

On planning the experimental technique it was assumed that the reaction between the soil and the added $CaCO_3$ would be more intense in a smaller volume of water, since the renewal of Ca-ion concentration from the solid $CaCO_3$ would be more rapid than would be possible with large volumes. Therefore, 30 ml additions of H_2O were used in each system, with renewal of that volume after each evaporation. If equally good results could be obtained from evaporation of an equal volume of H_2O added in larger additions, some time and effort would be saved. Results from reactions between $CaCO_3$ and twelve soils, with four 30 ml additions of water against a single 120 ml addition and evaporation, are given in Table 4. These findings show that with the exception of the highly adsorptive Portsmouth muck, reaction is advanced farther through repeated 30 ml inputs of water and evaporations than against a single 120 ml input and evaporation; the mean of the differences is 1.6 meq. The largest differences occurred in the soils of high clay content, regardless of the extent of reaction.

EFFECT OF NUMBER OF EVAPORATIONS ON REACTION PROGRESS USING LARGER NUMBER OF SOILS

Because the discussion of the results of Table 3 and Figure 1 were confined to four soils, it was deemed desirable to obtain information as to the speed and extent of the soil-$CaCO_3$ reaction on a larger number of

TABLE 4.—*Effect upon soil-$CaCO_3$ reaction of repeated evaporations (A), as compared with single evaporation of equal volume (B)*

SOILS	$CaCO_3$ ADDED	$CaCO_3$ REACTION		DIFFERENCE
		A[1]	B[2]	
	meq.	*meq.*	*meq.*	*meq.*
Hartsells fine sandy loam	30	21.5	19.6	1.9
Cumberland subsoil, 1950	20	13.1	11.4	1.7
Apison silt loam	20	5.5	4.6	0.9
Sequoia silt loam	20	10.0	9.0	1.0
Dickson silt loam	20	8.1	7.4	0.7
Hagerston silt loam	20	5.6	4.5	1.1
Carrington clay loam, B horizon	20	9.4	7.3	2.1
Talladega clay loam	25	15.8	13.2	2.6
Volusia silt loam	20	11.4	10.1	1.3
Wooster silt loam	20	10.0	8.8	1.2
Susquehanna clay subsoil	40	30.9	28.0	2.9
Mean difference				1.6

[1] A—Four evaporations to dryness of 30 ml H_2O.
[2] B—One 120 ml of H_2O evaporated to dryness.

soils, as well as additional data on the same four soils. The additional findings from experiments on thirteen soils are given in Table 5. The primary objective of these experiments was to determine the consistency of the division line on the reaction progress from the eight evaporations already discussed. Upon the basis of twelve comparisons, a mean of 1.42 meq. represented the increases induced by eight evaporations against values from four evaporations, whereas the mean increase from twelve evaporations against the eight evaporations was only 0.4 meq. Table 5 also includes results of twenty-four evaporations on a number of soils which were used in Figure 1.

The column headed "(4)+4" shows values obtained from combined evaporations of 120 ml of H_2O and four evaporations of 30 ml each. This was an attempt to accomplish the reaction attained in eight evaporations without extending the time to two days. It was thought that the overnight evaporation of 120 ml plus the four 30-ml evaporations might effect in one twenty-four-hour day what would require two working days. A comparison of the results obtained on thirteen soils by this technique as compared with those from the regular eight evaporations shows appreciable differences in only two or three instances. The mean deficiency by the shorter time was only 0.3 meq. This value is believed to be real, although statistically it is not significant. Better concordance might have resulted through reversal of the order, that is by first evaporating the four 30-ml additions during the day and the single 120-ml additions during the following night.

TABLE 5.—*Effect of number of evaporations on steam bath upon extent of reaction of soils with calcium carbonate*

SOIL TYPE	CALCIUM CARBONATE ADDED	NO. OF EVAPORATIONS (30 ML)				
		4	8	12	24	(4)+4*
	meq.	*meq.*	*meq.*	*meq.*	*meq.*	*meq.*
Hartsells sandy loam	30	21.4	23.3	23.9	26.0	23.1
1950		21.9	23.6	23.3	26.6	23.0
		21.3	23.1	24.1	26.0	22.6
Mean		21.5	23.3	23.8	26.2	22.9
Hartsells sandy loam	30	18.4	19.9		22.2	19.8
1951		18.0	20.1		22.5	20.2
		18.4	20.1		22.3	19.6
Mean		18.3	20.0		22.3	19.9
Cumberland clay	20	13.2	14.0	14.3	15.3	13.9
subsoil, 1950		13.3	14.2	14.5	15.2	14.1
		12.9	14.4	14.8	14.5	14.0
Mean		13.1	14.2	14.5	15.0	14.0
Susquehanna clay	40	30.6	32.9	32.5	32.1	31.7
subsoil, 1950		30.7	32.7	32.6	32.2	31.9
		31.3	32.6	32.7	32.7	31.8
Mean		30.9	32.7	32.6	32.3	31.8
Portsmouth muck	90	66.1	69.1	70.4	71.8	70.8
Florida, 1950		67.0	72.0	71.9	73.8	70.8
		67.0	73.0	74.4	73.4	73.0
Mean		66.7	71.4	72.2	73.0	71.5
Apison silt loam	20	5.2	5.7	5.8	6.6	5.1
		5.8	5.7	5.8	6.7	5.2
		5.6	5.9	5.7	7.0	5.7
Mean		5.5	5.8	5.8	6.8	5.3
Sequoia silt loam	20	9.9	11.1	11.3		9.9
		9.7	10.9	11.3		10.2
		10.3	10.8	11.2		10.2
Mean		10.0	10.9	11.3		10.1
Dickson silt loam	20	8.5	8.9	9.6		8.6
		8.1	9.2	9.5		8.3
		7.8	8.8	9.3		8.6
Mean		8.1	9.0	9.5		8.5

TABLE 5.—(*continued*)

SOIL TYPE	CALCIUM CARBONATE ADDED	NO. OF EVAPORATIONS (30 ML)				
		4	8	12	24	(4)+4*
Hagerston silt loam	20	5.5	6.7	6.7		6.6
		5.7	6.7	6.7		6.1
		5.6	6.2	6.8		6.0
Mean		5.6	6.5	6.7		6.2
Carrington silt subsoil	20	9.4	11.2	11.7		10.5
		9.6	11.0	11.5		11.2
		9.5	11.4	11.5		10.4
Mean		9.5	11.2	11.6		10.7
Talladega clay loam	25	15.8	16.6	17.4	19.5	17.0
		15.7	17.1	17.6	19.1	16.7
		15.9	17.3	17.7	19.3	16.9
Mean		15.8	17.0	17.6	19.3	16.9
Volusia silt loam	20	11.6	12.3	13.6		12.8
		11.4	12.7	13.7		13.1
		11.3	12.8	13.5		12.8
Mean		11.4	12.6	13.6		12.9
Wooster silt loam	20	9.9	10.6	11.1		10.6
		10.3	10.5	11.1		10.8
		9.9	10.5	11.0		10.7
Mean		10.0	10.5	11.1		10.7

* (4) +4 = 120 ml evaporated once +4 × 30 ml.

EFFECT OF SOURCE OF CARBONATE UPON REACTION PROGRESS

In an earlier study at this Station (26) it was noted that the progress of the soil-$CaCO_3$ reaction was influenced by the type of the precipitated carbonate. Because of that observation, it was deemed desirable to compare calcium carbonates other than the ones used in the present study.

The several types of calcium carbonates and their reactivities with three soils are given in Table 7; the findings are mean values of triplicate determinations. Examination of data for each soil and treatment shows a distinct grouping of materials with respect to their reactivities. In every instance, least reactivity was from the 1951 samples of J. T. Baker. The 325-mesh portion of that sample and the 325-mesh Iceland spar fall into the group of intermediate activity. The materials that show the highest and nearly equal reactivities are recent samples of precipitated carbon-

TABLE 6.—*Effect of rate of evaporation upon speed of $CaCO_3$ reaction with soil*

SOIL	$CaCO_3$ ADDED	REACTION AT EVAPORATION SPEEDS		
		A[1]	B[2]	C[3]
	meq.	*meq.*	*meq.*	*meq.*
Talladega clay loam	25.0	12.2	12.1	11.6
		11.8	11.4	12.0
		12.3	11.8	12.1
	(Av.)	12.1	11.8	11.9

[1] A—Beakers of boiling water, serving as steam bath, directly over hot plate burner, water boiling vigorously. (Required two hours for evaporation.)
[2] B—Beaker position: 10 inches away from A. Water 90 to 95°. (Required four hours for evaporation.)
[3] C—Beaker position: 15 inches away from A. Water 80 to 85°. (Required six hours for evaporation.)

ates from J. T. Baker and from Merck, and the 325-mesh marble. The lower part of Table 7 gives the mean reactivity values for three soils and treatments that were in common for those materials. The difference between extreme grades of reactivity is about 2.5 meq. per 100 grams of soil. This difference in reactivity may occur also between different lots of the same source, as can be seen in the results of the older and the recent

TABLE 7.—*Effect of source of calcium carbonate upon soil—$CaCO_3$ reaction at steam bath temperature*

SOURCE OF CALCIUM CARBONATE	HARTSELLS SANDY LOAMS			TALLADEGA CLAY LOAM		
	1950	1951				
	(4)+4	4	8	(4)+4	12	24
Iceland spar, 325-mesh	21.5	17.1	19.1	16.2	16.5	18.8
J. T. Baker, 1951, Low in alkalies	—	15.9	—	14.8	—	—
J. T. Baker, 1951, 325-mesh	21.3	—	18.8	—	16.5	18.8
J. T. Baker, 1953, Low in alkalies	23.5	18.2	—	17.5	—	—
Merck, 1953, Low in alkalies	23.4	18.5	—	17.1	—	—
Marble, 325-mesh	22.9	18.3	20.0	17.2	—	19.3

	Combined Mean Values for Three Soils, Nine Determinations Each
Iceland spar	18.3
Marble, 325-mesh	19.5
J. T. Baker, 1953	19.7
Merck, 1953	19.7

lots of J. T. Baker's precipitated calcium carbonate. Although these differences are being narrowed in the longer reaction periods, they are not completely erased even with the longest contact period of six days on the steam bath.

Remarkable differences appeared in the turbidities of the aqueous suspensions of the several carbonates. The more reactive calcium carbonates showed the greatest turbidity, and degree of turbidity is related to fineness of the carbonate. The appearance of the bulk sample may help in recognizing the fineness of particle size of the carbonate, one of finest particle size has a "curdy" aggregate appearance, whereas the one of coarser particle size is more powdery. The fact that three carbonates from different sources were found to have equal reactivity, indicates that it should not be difficult to duplicate results through the use of precipitated $CaCO_3$, especially when it is tested for fineness by means of turbidity tests.

EFFECT OF EXCESSES OF CARBONATE UPON EXTENT OF SOIL-$CaCO_3$ REACTION

In the determination of the calcium sorption capacities of silt and sandy loams, $CaCO_3$ was added at the rate of 20 meq. (one gram of $CaCO_3$) per 100 grams of soil. Under most circumstances, after reaction with the soils, a $CaCO_3$ residue ranging from 10 to 15 meq. will be found. Occasionally in cases of soils of high organic matter content, the $CaCO_3$ residue may be only 5 meq. or less. A previous investigation of Ca-sorption capacities of soils at 30°C. (44) demonstrated that an excess of 5 to 10 meq. of $CaCO_3$ was necessary for maximal values. In the present study of the reaction at 95°C., the quantity of $CaCO_3$ excess necessary for maximal reaction was investigated by supplying $CaCO_3$ to six typical soils in range of 5, 10, and 15 meq. above the previously determined Ca-sorption values. The results from four evaporations on steam bath are given in Table 8. Four of the soils were also supplied with $CaCO_3$ in exact equivalence of their determined Ca-sorption capacities.

From triplicate determinations for each condition for the soils (Table 8), the probable points of maximal Ca-sorption appear to be as follows: Hartsells soil, 5 meq. $CaCO_3$ excess; Norfolk soil, 5 meq. excess; Cumberland subsoil, 10 meq.; Talladega soil, 15 meq.; Baxter soil, 10 meq.; Tellico, 10 meq. The data indicate that a 10 meq. excess is most desirable for maximal values of Ca-sorption under the four-time wetting and drying steam bath procedure. The Ca-sorption in the presence of only 5 meq. excess has been found generally to be about 0.5 meq., and in no instance more than 1.0 meq., less than the sorption with 10 meq. excess with the carbonate additions equivalent to the determined Ca-sorption values only, the Ca-sorptions were from 1 to 2 meq. short of complete reaction. (This finding is in contrast to the complete $CaCO_3$ reaction noted when the addition was limited to the equivalent of exchangeable hydrogen indicated by the Ca-acetate procedure.)

TABLE 8.—*Effect of increasing excess of calcium carbonate upon soil-calcium carbonate reaction**

SOIL TYPE	REPLICATES	$CaCO_3$ ADDED—GRAMS PER 10G TO GIVE EXCESS IN MEQ. PER 100G:				$CaCO_3$ REACTION—MEQ./100G FROM APPROXIMATE EXCESSES OF:			
		0	5	10	15	0	5	10	15
Hartsells sandy loam	1	0.1025	0.1275	0.1525	0.1775	18.6	19.7	20.2	19.7
	2	.1025	.1275	.1525	.1775	18.4	19.7	20.6	19.9
	3	.1025	.1275	.1525	.1775	18.4	19.9	20.3	20.0
	(Av.)	.1025	.1275	.1525	.1775	18.5	19.8	20.4	19.9
Norfolk sandy loam	1	.0295	.0545	.0795	.1045	4.8	5.8	5.7	5.7
	2	.0295	.0545	.0795	.1045	4.8	5.5	5.5	5.9
	3	.0295	.0545	.0795	.1045	4.8	5.5	5.7	5.8
	(Av.)	.0295	.0545	.0795	.1045	4.8	5.6	5.6	5.8
Cumberland clay subsoil	1	.0675	.0925	.1175	.1425	12.1	13.7	14.5	14.3
	2	.0675	.0925	.1175	.1425	12.3	13.2	14.3	14.6
	3	.0675	.0925	.1175	.1425	12.2	13.6	14.1	14.7
	(Av.)	.0675	.0925	.1175	.1425	12.2	13.5	14.3	14.5
Talladega clay loam	1	.0730	.0980	.1230	.1480	12.6	14.4	14.7	15.6
	2	.0730	.0980	.1230	.1480	12.6	14.1	14.4	15.4
	3	.0730	.0980	.1230	.1480	12.6	14.4	15.0	15.9
	(Av.)	.0730	.0980	.1230	.1480	12.6	14.3	14.7	15.6
Baxter silt loam	1	—	.0695	.0945	.1195	—	7.5	7.9	8.2
	2	—	.0695	.0945	.1195	—	7.5	8.2	8.3
	3	—	.0695	.0945	.1195	—	7.6	8.0	7.8
	(Av.)		.0695	.0945	.1195		7.5	8.0	8.1
Tellico sandy loam	1	—	.0765	.1015	.1265	—	10.4	11.6	12.1
	2	—	.0765	.1015	.1265	—	10.7	11.3	11.6
	3	—	.0765	.1015	.1265	—	10.7	11.8	11.6
	(Av.)		.0765	.1015	.1265		10.6	11.6	11.8

* 4×30 ml evaporations on steam bath.

COMPARISON OF Ca-SORPTION VALUES OBTAINED THROUGH SEVERAL PROCEDURES

Earlier attempts have been made to establish the validity of the soil-$CaCO_3$ reaction at steam-bath temperature (a) through comparison with results obtained at 30°C. under nearly natural conditions during one year, and (b) through examination of reaction progress at 95°C. revealing clear-cut transitions from a decreasing rate of reaction to a reaction of near constant rate, as shown by straight line extension of the curves in Figure 1. The small but constant differences between the results at the two temperatures, and the difficulty of duplicating the long-time experiments made it advisable to check the performance of the Ca-sorption determination at 95°C. against other procedures. Values obtained by means of the Bradfield and Allison, Patel and Truog, and Mehlich procedures, which have

the common feature that their reaction media are slightly above *p*H 8, are given in Table 9.

It has been pointed out that the Bradfield and Allison procedure is rated as standard for this determination. The special apparatus prescribed by Bradfield and Allison was not available, and therefore, it was necessary to make certain that the technique used resulted in true equilibration. The three columns of data under "$Ca(OH)_2$-CO_2-air equilibration," Table 9, provide the answer. Sixteen-hour air passage with continuous agitation resulted in complete equilibration, because identical results were obtained after additional sixteen-hour air passage. However, eighteen-hour air passage through bubbling only, without agitation except for occasional shaking during the first two hours, gave results that were, on the average, 3 meq. short of equilibrium values that were obtained by continuous agitation.

TABLE 9.—*Comparison of calcium sorption results by several procedures, all at pH above 8*

(in meq. per 100 grams of soil)

NUMBER	SOILS	$Ca(OH)_2$-CO_2-AIR EQUILIBRATION (7)			$CaCO_3$ REACTION AT 95°C.	$Ca(HCO_3)_2$ REACTION (39)	TRIETHANOLAMINE-$BaCl_2$ (34)
		18-HOUR BUBBLING	CONTINUOUS SHAKING				
			16 HRS.	32 HRS.			
1	Hartsells sandy loam, 1950	21.0	24.6	24.6	23.5	—	—
2	Talladega clay loam	15.5	17.5	17.5	17.5	13.0	12.5
3	Cumb. clay subsoil	9.4	12.8	13.0	13.2	10.3	11.5
4	Wooster silt loam	7.8	11.5	11.5	10.7	10.0	10.7
5	Norfolk sandy loam	5.2	6.6	6.5	6.6	—	4.1
6	Volusia silt loam	10.5	—	—	12.9	11.7	8.5
	Mean (1–5)	11.8	14.6		14.3		
	Mean Diff.	2.8	—		0.3		

The departure from the original technique consisted in the utilization of 125-ml extraction flasks, provided with inlet and outlet tubes through No. 7 stoppers, and connected in series. Continuous agitation was secured by means of a Ross-Kershaw shaker. To ensure continuous operation, the stoppers were wired to the flasks and the flasks fastened to the clamps with strong rubber bands. The air equilibration took place during the night with laboratory air which was washed through two bottles of distilled water. There was no gas flame in the laboratory during that time, and the air was considered the same as that from the outside. The air current was produced by an electric vacuum pump, and air flow was regulated by valve between the pump and the soil systems. Because the

lower end of the inlet tubes accumulated carbonate deposits, these parts were connected by rubber tubing that could be disconnected readily so that the parts could be retained in the flask for carbonate analysis. To avoid correction for dissolved bicarbonate after equilibration, the suspensions were evaporated to dryness on a steam bath, and the residual carbonate was determined by the steam distillation procedure (43). Where the shaker was used, the volume of the $Ca(OH)_2$ solution had to be restricted to 50 ml to avoid splashing of soil suspension into the outlet tubes. In using the Bradfield-Allison procedure for soils of Ca-sorptions greater than 15 meq., it was found necessary to decrease the soil charge to below 10 grams. In comparison with the air-equilibration values, the mean of the $CaCO_3$ reactions at 95° (five soils) was 0.3 meq. lower. These results demonstrate that soil-$CaCO_3$ reaction on the steam bath exerts no deleterious effect upon the soils, and that the earlier conclusion, viz., that the eight 30 ml evaporations on the steam bath assures completion of the main reaction, is substantiated by the $CaCO_3$-air equilibration results. It would be desirable to obtain further support of this conclusion through correspondingly good agreement on a larger number of soils.

If the results on a larger number of soils by the two procedures should prove to be in equally good agreement, the described soil-$CaCO_3$ reaction procedure would have a number of advantages, namely: a constant soil charge can be used regardless of the Ca-sorption capacity of the soil; there is no need for special reaction flasks, shaking device, and connections; and the only stock reagent needed is $CaCO_3$ of high purity.

The results by both the triethanolamine-$BaCl_2$ and the $Ca(HCO_3)_2$ procedures appear in most instances to fall short of the equilibration values given in Table 9.

SUMMARY

This investigation deals with the determination of the speed of the reaction of soils and incorporated calcium carbonate under various conditions, both at room temperature (30°C.) and at 95°C., with a view to establishing a laboratory procedure for the determination of the soils' potential capacity for calcium sorption under continued contact with $CaCO_3$. Since the reaction between soils and calcium carbonate and calcium bicarbonate have been frequently used in the determination of the "lime requirement" of soils, it was felt necessary to give a review of concepts, and appraisal of procedures developed during fifty years. The strictly chemical procedures that were proposed in the first twenty years of the century were not successful; they either failed to register maximal reaction values, or they gave lime requirement indications that were excessive in relation to practical liming for crop production. The application of physico-chemical measurements to the soil-$CaCO_3$ reaction system, begun by the Danish chemists in 1919, and perfected by Bradfield and Allison

in 1933, led to a new concept of a "base saturated" soil, based on soil-$CaCO_3$-H_2O-air equilibration. Bradfield and Allison also devised a procedure for the determination of the saturation deficit, or exchangeable hydrogen of the soil, which, when added to the exchangeable base content gave the soil's saturation value.

Four types of soils were supplied with an excess of 325-mesh marble and incubated for various periods from two weeks to one year at 30°C. and the resultant decompositions established the rate of reaction under nearly natural conditions. When the same soils received an excess of calcium carbonate and were subjected to four to twenty-four evaporations with 30 ml of water on the steam bath, a transition in the rate of reaction was indicated at the point of eight evaporations. Nearly equal results were obtained through evaporation of four 30-ml portions of water and one evaporation of 120 ml in a twenty-four-hour contact as were obtained on eight evaporations requiring two working days. By this treatment the soil-$CaCO_3$ reaction was advanced to the extent attained in six months at room temperature. The results obtained from twenty-four digestions of five soils with an excess of $CaCO_3$ on the steam bath were compared with results obtained by the Bradfield and Allison $CaCO_3$-equilibration procedure. With the use of plain flasks, an eighteen-hour air bubbling was not sufficient to establish equilibrium, but equilibrium was established in sixteen hours of air bubbling with gentle shaking on a Ross-Kershaw shaker. The mean value for five soils from evaporation was only 0.3 meq. below that determined by $Ca(OH)_2$-CO_2-air equilibration. The close agreement of the results on five soils by such different procedures, especially with respect to the temperatures of 30°C. and 95°C., is believed to demonstrate the significance and validity of the results obtained by both procedures. Comparison of results on a larger number of soils of greater variety would be advantageous. The steam bath procedure has certain practical advantages and promises to become an expeditious routine procedure for the determination of the saturation deficit, or the calcium sorption capacity of soils, when the exchangeable base content has been added to it.

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RAPID DETERMINATION OF CATION AND ANION EXCHANGE PROPERTIES AND *p*He OF SOILS*

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The characterization of soils from the standpoint of exchange properties involves principally the determination of cation exchange capacity, anion exchange capacity, exchangeable H, Ca, K, and Na, and exchangeable PO_4. The value of a method, usable on all soils, regardless of type of colloid present or the base status, cannot be underestimated. It is important also to select a method whereby the principal cations and the cation exchange capacity are determined using the same extractant. This has the advantage that the sum of the exchangeable cations can be compared with the cation exchange capacity. Such information can be used to check on the accuracy of the analysis, since, under ideal soil conditions, the sum of cations should be the same as the cation exchange capacity. Whenever the analysis is found to be accurate and the sum of cations exceeds the cation exchange capacity it may be assumed that the soil contains salts, free acids, or free bases in addition to the exchangeable cations. A knowledge of any of these conditions is essential for an accurate characterization of the exchange properties of soils.

The barium chloride-triethanolamine method of extraction (4, 6, 7, 9) meets most of these requirements. It can be used successfully on all soils, regardless of soil type and/or base status. In view of the importance of a knowledge of the cation and anion exchange status of soils in problems of liming, fertilization, and soil classification, it is essential to select a method which is rapid in order that a large number of soils may be economically tested. Experience with the barium chloride-triethanolamine method has shown that the various specific procedures can be rendered more rapid, without loss of accuracy, than those described previously (9).

The procedures involve the volumetric and flame photometric determination of Ca, the volumetric determination of H, the colorimetric determination of Mg and the flame photometric determination of K and Na. In addition, procedures are given for the determination of cation exchange capacity involving the determination of Ba by colorimetric and flame photometer techniques. Methods are also described for the measurement of anion exchange capacity, exchangeable P, and *p*He. Consideration is also given to the significance of *p*He and cation exchange capacity-anion exchange capacity ratios (C/A), in relation to the proximate identification of mineral colloids in soil.

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METHOD

REAGENTS

(a) *Barium chloride-triethanolamine.*—Dilute 90 ml of commercial triethanolamine (sp. gr. 1.126) with 1 liter of H_2O and adjust to *p*H 8.1 with HCl. This requires about 280 to 300 ml of 1 *N* HCl. Make up to 2 liters with H_2O and mix with 2 liters of a soln containing 100 g of $BaCl_2.2H_2O$. Protect from CO_2 during storage.

(b) *Barium chloride, 0.1 N.*—Dissolve 12.22 g $BaCl_2 \cdot 2H_2O$ and make to 1 liter with H_2O.

(c) *Mixed indicator.*—Triturate 0.1 g of bromcresol green indicator with 16 ml of 0.01 *N* NH_4OH and dilute to 200 ml with H_2O. Dissolve 0.1 g of methyl red in 200 ml of 95% ethanol. Mix equal volumes of these indicators before use.

(d) *Ba and Ca ppt reagent.*—Dissolve 20 g oxalic acid, 50 g ammonium acetate and 50 g $(NH_4)_2SO_4$ and make up to 1 liter with H_2O.

(dd) *Ba ppt reagent.*—As (d) but omit oxalic acid.

(e) *Calcium chloride.*—50 g $CaCl_2 \cdot 2H_2O$ per liter of H_2O. Adjust to ca *p*H 8 with satd $Ca(OH)_2$ soln.

(f) *Li soln—2000 p.p.m.*—12.22 g LiCl per liter of H_2O.

(g) *Thiazol yellow.*—Dissolve 0.1 g of thiazol yellow and make up to 500 ml with H_2O. Store in refrigerator and make up fresh every two months.

(h) *Phosphoric acid, 0.03 N.*—Measure 1.35 ml of 85% H_3PO_4 into a 2-liter volumetric flask and make up to volume with H_2O.

(i) *Ammonium vanadate–ammonium molybdate soln.*—Weigh 1.25 g NH_4VO_3 into a 500 ml volumetric flask. Add 400 ml of 1:2 (by volume) HNO_3, heat slightly to dissolve, cool, and make to vol. with 1:2 HNO_3. Dissolve 25 g ammonium molybdate in H_2O and make up to 500 ml with H_2O. Mix equal volumes of these solns before use.

(j) *Stock soln of Ca, Mg, K, and Na salts.*—Dissolve 3.6758 g $CaCl_2 \cdot 2H_2O$, 2.0333 g $MgCl_2 \cdot 6H_2O$, 0.1491 g KCl, and 0.1169 g NaCl and make up to 1 liter with H_2O.

(k) *Standard solns.*—Add from 0 to 10 ml of stock soln (reagent j) into 200 ml beakers contg 20 ml of reagent (a), 20 ml of reagent (b), 25 ml of H_2O, and 6 drops of mixed indicator. Carry through detn as described under H, Ca, etc. (1 ml of the stock soln is equivalent to 0.05 meq. Ca, 0.02 meq. Mg, 0.002 meq. K, and 0.002 meq. Na).

(l) *Ba standards for cation exchange capacity.*—Measure from 0 to 12 ml of reagent (b) into 100-ml volumetric flasks contg 50 ml of reagent (e). Make up to vol. with H_2O and proceed as directed under "Cation Exchange Capacity."

(m) *Phosphate standard for anion exchange capacity and exchangeable phosphate.*—Measure from 0 to 1 ml of reagent (h) into vials, and dilute with H_2O to 20 ml. Add 2 ml of reagent (i), mix, and after 15 minutes measure transmission at 425 mμ. Plot instrument reading against phosphate additions as follows: For anion exchange capacity: 0 ml. = 3.0, 0.2 ml. = 2.4, 0.4 = 1.8. . . . 1.0 ml. = 0. For exchangeable PO_4:0 ml = 0, 0.2 ml. = 0.3, 0.4 ml. = 0.6 . . . 1.0 ml. = 1.5.

(n) *HCl-NH_4F soln for exchangeable phosphate.*—Dissolve 2.25 g NH_4F in a liter of 0.05 *N* HCl. The resulting soln is 0.06 *N* NH_4F at *p*H 3.0.

(o) *HCl, 0.04 N.*

SPECIAL EQUIPMENT

(a) *Special leaching tubes* with perforated porcelain disk permanently mounted. (Obtainable from Southern Scientific Co., Inc., Atlanta 3, Georgia.) Specifications: Over-all length, 150 mm; stem length approx 75 mm; inner diam. of tube 32 mm; perforated porcelain disk 23 mm to take a 21-mm diam. filter paper circle.

(b)* *Special filter stand* for holding the leaching tubes, flasks or beakers in units of 10.

(c)* *Automatic pipettes*, 20 ml capacity, in units of 10 for the delivery of solutions (a), (b), and (h).

DETERMINATIONS

Place a 21 mm diam. filter paper on the perforated porcelain disk of the special leaching tube, moisten with H_2O, and draw into place with suction or pressure. Weigh an amount of 1 mm sieved air-dry soil to give 0.2 to 1.0 meq.† cation exchange capacity into the tubes, level soil, and place a 30-mm filter paper on top. Collect leachates in 200 ml beakers. Add 20 ml of replacement soln (a) and, after draining, 20 ml of soln (b). After complete draining, wash 6 times with 8–10 ml portions of H_2O. Rinse stem of filter tube into beaker to remove Ba (due to splashing). Save soil and proceed as under "Cation Exchange Capacity."

Hydrogen.—Titrate the ext. with 0.04 *N* HCl to a pale rose, using 6 drops of mixed indicator. Titrate likewise a mixture of 20 ml each of solns (a) and (b). The difference between these two titration values in ml, multiplied by 4, and divided by the wt of soil, is equal to meq. H per 100 g of soil.

Calcium-A (volumetric, in presence of Ba).—After titrating for H, place beaker on hot plate and heat to 80–90°C. Add with stirring 10 ml of soln (d). Digest for 1 hour or until clear. Decant the supernatant liquid and then the ppt through 30 ml Selas microporous filter crucibles into 200 ml volumetric "phosphoric acid" flasks. Wash the beaker and then the ppt with six 5–6 ml portions of H_2O.

Rinse outside crucible, return crucible to beaker, dissolve ppt with about 100 ml hot *N* H_2SO_4, and titrate with .025 *N* $KMnO_4$. (Ml 0.025 *N* $KMnO_4 \times 2.5$ divided by wt of soil = meq. Ca per 100 g soil.) Standardize $KMnO_4$ against known amounts of Ca (see reagent k) carried through above procedure.

Calcium-B (volumetric following removal of Ba).—After titrating for H, add with stirring 10 ml of soln (dd), place on hot plate, and digest for 1 hour or until clear. Decant the supernatant liquid, and then the ppt, through 30 ml Selas Microporous filter crucibles into 200-ml beakers. Wash the beaker and then the ppt with six 5–6 ml portions of H_2O.

Place on hot plate and heat to 80–90°C. and add with stirring 10 ml of 2% oxalic acid soln. Then proceed as under Calcium-A.

Calcium-C (flame photometric).—Proceed as under calcium-B but collect filtrate in 200 ml volumetric "phosphoric acid" flasks. Add 10 ml of reagent (f) (omit if K and Na determined by direct method) and make up to volume with H_2O. Det. Ca by means of a flame photometer at a wave length of 554 mμ. (In the development of the present method, a Beckman Model B instrument was used.) Prepare a calibration curve from the standard solns (reagent k) carried through in the same way as the unknown and express results as meq. Ca per 100 g soil.

Magnesium (Colorimetric).—Add to the filtrate from the Ca ppt (A or B) 10 ml of reagent (f) (omit if K and Na determined by direct method) and make up to volume with H_2O. Pipet 20 ml of the filtrate from Calcium-A or B into a vial or flask; add 1 ml of reagent (g), mix, add 2 ml of 30% NaOH (low in carbonates), mix, and within 2 min. measure the transmission of the soln at 525 mμ. Use a water blank for the 0 setting of the instrument. If concn of Mg is too high, use a 5 ml aliquot and dilute with 15 ml of H_2O. Convert readings into concentration of Mg by comparison with a calibration curve constructed from standard solns (reagent k) of Mg and carried through the regular procedure. Derive also 2 sets of standard

* Both (b) and (c) are obtainable from the Soil Testing Equipment Co., Box 64, Station A, Ames, Iowa.

† Use 8 g for sands; 4 g for sandy loams, silt, or clay loams; 2 g for clay; and 1 g for muck and peat.

curves, one using 20 ml and another using 5 ml diluted with 15 ml H_2O. Express as meq. Mg per 100 g soil.

Potassium (flame photometric).—Use the filtrate from Calcium-A or -B for the flame photometric detn of K. Locate the position of the K line with the standard soln contg 10 ml of stock soln (reagent j). Compare the instrument reading of the unknown against a standard curve prepared with reagent (k). Express results as meq. K per 100 g soil. If the concn of K in the unknown is too high, dil. with standard soln (prepd. with 0 addn of stock soln) and multiply by the corresponding soln factor.

Sodium (flame photometric).—Use the filtrate from Calcium-A or -B for the flame photometric detn of Na. Find the Na line with the standard soln contg 10 ml of stock soln. Compare the instrument reading against a standard curve prepd. with reagent (k). Express results as meq. per 100 g soil. If the concn of the unknown is too high, dilute with standard soln (0 addition of stock soln) and multply by the corresponding diln factor.

CATION EXCHANGE CAPACITY

Leach the Ba-soil in the filter tube with 50 ml of reagent (e). Collect leachate in a 100 ml volumetric "Bates" flask. Wash 5 times with 7–8 ml of H_2O. Wash the stem of the tube into flask and make up to vol. with H_2O.

Colorimetric determination of Ba.—Measure 10 ml portions of the leachate into 15 ml centrifuge tubes, add 1 ml of 10% K_2CrO_4, mix and place in a water bath heated to 80–90°C. After 20 to 30 min., shake tubes gently to cause coatings on top to settle. After 1 hour, cool to room temp. and centrifuge for 15 min. at 1700 r.p.m. Decant, rinse the mouth of tube, and then add about 5 ml satd $BaCrO_4$ soln in such a way as to break up the precipitate. Again centrifuge, decant, allow to drain, and dissolve the ppt with 10 ml dilute HCl (1:4 by volume). Measure the transmission at 425 mμ, using a water blank for the 0 setting. Obtain the meq. Ba from curve interpolation of standard Ba solns prepared as above (see reagent 1). Express as meq. per 100 g soil.

In the development of the present method, a Fisher electrophotometer was employed. By plotting the log readings (absorbance) against Ba concn, a straight line was obtained. It was thus possible to employ factors for converting log readings into meq. cation exchange capacity per 100 g soil. These factors were: for 8 g soil, 0.33; for 4 g soil, 0.66; for 2 g soil, 1.32.

Flame photometric determination of Ba.—Use propane as fuel and a red sensitive photocell. Find the Ba line on the wave length scale at 873 mμ using the Ba standard soln contg 12 ml of 0.1 N $BaCl_2$ (reagent 1). Balance the instrument with the 0 and 12 ml $BaCl_2$ standards at 0 and 100 respectively. After every 4 to 6 samples, check the adjustment with one of the $BaCl_2$ standards nearest the last reading. Report results as meq. cation exchange capacity per 100 g soil by multiplying the concn of Ba found (viz., 0 to 1.2 meq. per 100 ml) by 12.5, 25, and 50 when the amount of soil used was 8, 4, and 2 g, respectively.

ANION EXCHANGE CAPACITY AND *p*He

Dry the Ca-soil (from cation exchange detn) in an oven at 45°C. Weigh out an amount of Ca-soil to give 0.20 meq. cation exchange capacity (20/C.E. cap.) and place into 50 ml Lusteroid centrifuge tubes. Add 20 ml of 0.03 N H_3PO_4, shake for 30 min., allow to stand 20 to 24 hrs., and again shake for 30 min. Detn the *p*H of the suspension. Report as *p*He. (This can be determined directly in tube when using an instrument with external electrodes.)

Either centrifuge at 2400 r.p.m. for 15 min. or filter; measure 1 ml of the supernatant liquid into a vial, and add 19 ml of H_2O and 2 ml of reagent (i). Mix, and after

15 min., read the transmission at 425 mμ. As a blank use 20 ml of H_2O and 2 ml of reagent (i). Express results as meq. anion exchange capacity per 100 g soil. Plot instrument readings against the ratio of PO_4 retained to cation exchange capacity, viz., 1 ml of reagent (h) = 0; 0.8 ml = 0.6; 0.6 = 1.2 and 0 = 3.0. Multiply the ratio found by the cation exchange capacity and add meq. of exchangeable PO_4. (If photometer readings are plotted against meq. PO_4 per sample, multiply by 100/wt of soil and add exchangeable PO_4 to obtain meq. anion exchange capacity per 100 g soil.)

EXCHANGEABLE PHOSPHORUS

Weigh 2 g of soil into a 50 ml Lusteroid tube or flask, add 1 scoop (ca 250 mgm) of Darco G60 charcoal, and then add 20 ml of reagent (n); shake for 30 min., and either centrifuge at 2400 r.p.m. for 15 min. or filter. Measure 10 ml of the clear centrifugate or filtrate into a vial, add 2 ml of reagent (i) and after 15 min read the transmission at 425 mμ. As a blank, use 10 ml of the extg soln and 1 ml of reagent (i). Express results in meq. PO_4 per 100 g soil from calibration data (see reagent (m)). Plot instrument readings against meq. PO_4 per 100 g soil.

PRELIMINARY TREATMENT OF SOILS CONTAINING CARBONATES

The characterization of soil from the standpoint of its equilibrium *p*H and the anion-exchange capacity by the proposed procedures requires a soil free of carbonates. Test for effervescence by treating the original soil with HCl. The soil showing evidence of carbonates is treated as follows: weigh 10 g portions of soil into 300 ml Erlenmeyer flasks, add 200 ml of 2 *N* NH_4Cl, and heat to boiling 1 to 2 hours or until no NH_3 perceptibly volatilizes. Add more NH_4Cl if necessary. Filter through a Büchner funnel, wash with copious amounts of H_2O, leach with 50 ml of reagent (a), then with 50 ml of reagent (b), and finally with 100 ml of H_2O. Dry, mix, weigh out a suitable amount of soil, replace the Ba with $CaCl_2$, and determine the cation-exchange capacity as previously described. Dry the Ca-soil, crush the soil particles, weigh out an amount to give 0.2 meq. cation exchange capacity, and det. anion exchange capacity and *p*He as described previously.

DISCUSSION

The advantages of the barium chloride-triethanolamine extracting solution are the following:

1. The employment of a divalent ion allows efficient replacement of metal cations.

2. The replacement of H is facilitated and quickly brought to completion due to the strong buffer properties of triethanolamine at *p*H 8.1 (7, 9). The reaction is as follows:

$$2H\text{-soil} + BaCl_2 \rightarrow Ba\text{-soil} + 2HCl \text{ and } 2HCl + 2(CH_2CH_2OH)_3NH^+OH^- \rightarrow 2(CH_2CH_2OH)_3NHCl + 2H_2O$$

3. The solubility of alkaline earth carbonates in the presence of barium chloride-triethanolamine is small (4, 8). The exchangeable cations (including H) can therefore be accurately determined in their presence. Free bases, notably alkaline carbonates, can be estimated from the excess titration over the "blank" solutions with HCl (see hydrogen). The presence of free acids (generally arising from the hydrolysis of aluminum) in highly base unsaturated soils can be estimated from the excess of the sum of the exchangeable cations and cation exchange capacity.

4. The adjustment of the buffer to *p*H 8.1 not only renders efficient the neutralization of exchangeable H, but it also reduces the chances of Ba forming insoluble salts (4, 8, 9).

EXCHANGEABLE CATIONS

Hydrogen.—The method for determining the exchangeable H has been found to give satisfactory results. In comparison with other methods employing a buffer medium, Innes and Birch (5) found $BaCl_2$-triethanolamine to replace H efficiently. Its high buffer capacity and sufficiently high *p*H give good results with soils of the 1:1 and organic types, which are known to require a higher *p*H for the effective neutralization of H (8).

Calcium.—The separation of Ba from Ca and subsequent volumetric determination of Ca is obviously time-consuming. Hence, if Ca is to be determined volumetrically it may be done more effectively in the presence of Ba. It is important to standardize $KMnO_4$ against known amounts of Ca carried through the regular procedures.

Although it has been observed that Ca can be determined flame photometrically in the presence of the concentration of Ba employed in the procedure, the removal of Ba at this stage is recommended. This is based on convenience, since in any event it is essential to remove Ba prior to the determination of K and Na. The addition of LiCl (reagent f) should be omitted if the direct method for the determination of K and Na is to be used.

Magnesium.—The exchangeable Mg is colorimetrically determined with thiazol yellow. The oxalate and other ions in the filtrate from the Ca determination do not interfere with the test. If, however, Mg is determined in the presence of Ca (calcium-C), this element should be compensated for by the addition of Ca. The error, however, is not great (see Table 3). The success of the method requires careful and accurate manipulation but depends principally on the purity of the dye. A product specially developed for the determination of Mg is now available on the market and has proved to be satisfactory.

Potassium.—The flame photometric determination of K is conveniently and rapidly accomplished following the removal of Ba or both Ba and Ca. In the development of the procedure a Perkin-Elmer Model 52A instrument has been predominantly employed. It has been checked also with a Beckman Model B flame photometer. With the Perkin-Elmer flame photometer the use of Li as the internal standard has given more reproducible results than direct reading (12, 13). The concentration of Li used was found to be an important factor in these determinations, although the human element in the operation was found to give the greatest variability (12, 13). Although the addition of Li has been included in the general procedure it is obviously to be omitted when direct measurements are made, as it interferes strongly in the direct method for both K and Na, particularly if acetylene is used as fuel.

The colorimetric method using Nitroso-R salt, which was described previously (9), has been omitted from the present procedure. The assumption was made that most laboratories now have access to a flame photometer whose use renders the determination of this element more rapid. If, however, it is necessary to employ a chemical procedure, its determination can be made colorimetrically in a replicate sample of the filtrate from calcium-B (prior to the determination of Ca). If this technique is used the ammonium must first be removed by evaporation in the presence of a slight excess of NaOH. The method previously described (9) is then followed.

Sodium.—The remarks made with respect to K apply in large part to the determination of Na. With the Perkin-Elmer Model 52A flame photometer, the internal Li standard method gives more reproducible results than the direct method. With the acetylene flame, Ca will affect the results some extent; therefore Na is determined more accurately if procedure "Calcium-A" is followed. Special precautions should be taken to avoid contamination, notably from glassware.

The chemical method for Na, using uranyl magnesium acetate, can be used on a replicate sample after the removal of Ba under "Calcium-B" (prior to the determination of Ca). After evaporation of the filtrate the procedure described previously (9) may be followed.

CATION EXCHANGE CAPACITY

The adsorption of Ba by soils in the presence of triethanolamine primarily involves exchange positions. This restriction, however, is not complete (7, 9). In avoiding the replacement of Ba from non-exchangeable sources, $CaCl_2$ has been found preferable to neutral NH_4OAc or HOAc (9). It was found, in addition, that 50 ml of neutral, normal NH_4OAc was less effective in the replacement of exchangeable Ba than an equivalent volume of 0.6 *N* $CaCl_2$. Some of these results are shown in Table 1. CaOAc had been used for comparison. It was found, however, that the acetate anion interfered with the precipitation of Ba as $BaCrO_4$ while the Cl anion did not interfere.

The alternate method of determining Ba (flame photometrically in the presence of Ca) can be successfully carried out by using a propane flame and a red sensitive phototube at a wave length of 873 mμ. With an acetylene flame, however, Ba cannot be determined in the presence of Ca at either 515 or 873 mμ (16). Ba can be measured under these latter conditions by using the acid-dissolved $BaCrO_4$ from the colorimetric procedure.

OPTIMUM CONDITIONS FOR THE FLAME PHOTOMETRIC DETERMINATION OF BA, CA, K, AND NA

The flame photometer characteristics needed for best results have been summarized in Table 2. Attention is called to the fact that although the use of Li as an internal standard reduces the effect of interfering ions, the error is not eliminated even if the ratio of the ion to be determined to the

TABLE 1.—*Cation exchange capacity, anion exchange capacity, pHe and organic matter of various soils*

SOIL TYPE	STATE	CATION EXCHANGE CAPACITY		ANION EXCHANGE CAPACITY	C/A	*p*He	O.M.
		NH_4OAc	$CaCl_2$				
		meq.	*meq.*	*meq.*			*per cent*
Cinabar. s l	Wash.	19.0	20.9	45.6	0.5	5.0	5+
Cecil. s l	S. C.	2.5	3.2	4.2	0.8	4.1	1.0
Cecil. l	N. C.	5.2	6.4	8.0	0.8	4.4	1.9
Lloyd. cl l	Ala.	10.0	11.2	14.0	0.8	4.7	2.0
Davidson. si cl l	Va.	11.6	15.7	16.5	1.0	4.5	3.3
Orangeburg. s l	Ga.	3.2	4.3	4.3	1.0	4.0	1.7
Chester. si l	Md.	8.1	9.9	9.1	1.1	4.0	2.3
Caribou. l	Maine	16.2	19.2	16.6	1.2	4.0	4.2
Norfolk. l s	N. C.	1.8	2.5	2.0	1.3	3.6	1.1
Hunt. cl	Miss.	25.0	29.2	8.4	3.5	3.4	2.7
Ft. Collins. l	Colo.	16.5	17.5	10.5	1.7	3.3	1.5
Fox. si l	Mich.	8.2	9.0	4.9	1.8	3.6	1.9
Muscatine. si l	Ill.	14.6	16.8	9.3	1.8	3.5	1.1
Elliot. si l	Canada	16.8	25.2	13.3	1.9	3.5	3.8
Moccasin. gr cl l	Mont.	20.2	26.1	11.4	2.3	3.4	2.8
Havillah. l	Wash.	20.2	24.4	10.0	2.4	3.4	5.0
Moody. si l	Nebr.	17.8	19.3	7.6	2.5	3.2	3.6
Carrington. l	Iowa	20.0	29.7	12.1	2.4	3.4	5+
Miami. si l	Wisc.	10.8	12.4	5.0	2.5	3.6	2.1
Lake Charles. si cl l	Texas	23.5	26.4	10.2	2.6	3.0	1.8
Barnes. s l	Minn.	28.8	34.0	10.6	3.2	3.0	5+
Barnes. l	S. D.	20.0	29.4	6.7	4.4	3.3	4.2
Draper. l	Utah	11.9	15.2	3.6	4.2	3.1	2.6
Langdon. l	N. D.	24.3	29.4	6.7	4.4	3.3	4.2

TABLE 2.—*Flame photometer characteristics for the determination of K, Na, Ca, and Ba*

ELEMENT	WAVE LENGTH	PHOTO TUBE	P.E.—52A[1]		BECKMAN—B[2]	
			INTERNAL STANDARD	COARSE SETTING	SENSITIVITY SETTING	APPROXIMATE SLIT (MM)
K	768	Red	Yes	6	3	0.6
Na	589	Red	Yes	4	—	0.4
		Blue	—	—	3	
Ca	554	Blue	—	—	4	0.8
Ba[3]	873	Red	No	4	3	1.1

[1] With the Perkin Elmer Model 52A a propane flame only was used. The pressures used were 10 and 5 lbs. for air and fuel, respectively.
[2] With the Beckman Model B, acetylene only was used. The pressure of O_2 used was 16 lbs. and that of C_2H_2 was 3 lbs. for K and 4 lbs. for Na, Ca and Ba.
[3] The settings for the Beckman refer to the determination of Ba following its separation from Ca, while those for the Perkin Elmer refer to the measurement of Ba in the presence of Ca.

interfering ion is large. (In a sample containing 20 p.p.m. K and 200 p.p.m. Na, the determined values were 42 and 98 p.p.m. K for the internal standard and direct method, respectively.) A correct analysis can be obtained only if the standards contain approximately the same concentration of interfering ions that are present in the unknown. However, if the cation concentration is in the range generally encountered in soils, the interference is small (Table 3).

ANION EXCHANGE CAPACITY AND *p*He

Since the proposed procedure involves an equilibrium reaction and the phosphorus adsorbed is determined by difference, a more accurate measure of anion exchange capacity is obtained if the NH_4F-replaced PO_4 is added to the PO_4 adsorbed. This procedure will correct for the presence of

TABLE 3.—*Interference of cations on K, Na, Ca and Mg*

INTERFERING CATIONS	CONCENTRATION	CATION DETERMINED*			
		Ca	K	Na	Mg
	meq.	*meq.*	*meq.*	*meq.*	*meq.*
None	0	12.5	0.50	0.50	5.0
Ca	12.5	—	.50	.50	5.4
K	0.5	12.8	—	.53	5.0
Na	0.5	12.7	.51	—	5.0
None	0	2.5	.10	.10	1.0
Ca	12.5	—	.10	.10	1.1
K	0.5	2.6	—	.104	1.0
Na	0.5	2.4	.104	—	1.0

* Ca, K and Na were determined by means of the Beckman Model B spectrophotometer, while Mg was determined colorimetrically with thiazol yellow.

Ca- and Ba-phosphates, which are rendered partly soluble by H_3PO_4 or by acidified NH_4F. This problem has been investigated by adding increasing amounts of phosphorus to Kamec halloysite and several kaolinitic soils. Following the removal of non-adsorbed phosphorus by leaching with H_2O, the samples were dried and the cation and anion exchange capacity and the exchangeable PO_4 determined. The results obtained are presented in Table 4. The data show that the anion exchange capacity decreases with increasing amounts of added phosphorus. However, when these values are corrected by adding the exchangeable PO_4, the anion exchange capacity values are reasonably constant. As a result of phosphating, the cation exchange capacity increases; this results in increased C/A ratios and lower *p*He values. Under the proposed procedure, the anion exchange capacity is determined at a variable *p*H. This variability is induced by the fact that the concentration of H_3PO_4 used (first H) is equivalent to the cation exchange capacity of a Ca-saturated soil. Hence,

TABLE 4.—*Effect of phosphating soils and clays on the anion exchange capacity (A.E.C.), cation exchange capacity (C.E.C.), and pHe*

PO_4 ADDED	PO_4 ADSORBED	EXCHANGEABLE PO_4	A.E.C.*		C.E.C.	C/A	*p*He
			NOT CORRECTED	CORRECTED			
meq.†	*meq.*	*meq.*	*meq.*	*meq.*			
			Kamec Halloysite				
0.0	0.0	0.3	13.2	13.5	7.4	0.5	4.0
3.7	3.4	2.5	11.8	14.3	9.0	0.6	4.0
7.4	4.8	4.7	8.9	13.6	10.2	0.8	3.9
14.8	7.4	5.5	8.2	13.7	11.3	0.8	3.8
			Nipe				
0.0	0.0	0.2	27.8	28.0	12.5	0.4	5.3
6.3	6.2	1.2	27.3	28.5	19.1	0.7	4.5
12.5	11.2	4.7	24.6	29.3	24.1	0.8	4.2
25.0	17.1	6.0	23.2	29.2	26.1	0.9	4.1
			Orangeburg				
0.0	0.0	0.2	7.3	7.5	4.2	0.6	3.7
2.1	2.0	0.9	6.9	7.8	5.0	0.6	3.5
4.2	2.9	1.9	5.0	6.9	5.5	0.8	3.5
8.4	4.2	2.4	4.8	7.2	6.0	0.8	3.4
12.6	6.9	2.7	4.7	7.4	6.4	0.9	3.2
			Susquehanna				
0.0	0.0	0.2	27.7	27.9	22.9	0.8	3.3
11.5	8.2	2.7	25.7	28.4	29.2	1.0	3.2
22.9	9.6	3.9	24.1	28.0	29.5	1.1	3.1
45.8	15.3	4.9	23.3	28.2	29.5	1.0	3.1
68.7	29.9	5.8	22.2	28.0	30.1	1.1	3.1

* The data under "not corrected" corresponds to the meq. PO_4 retained and that under "corrected" corresponds to the meq. PO_4 retained plus meq. of PO_4 exchanged.
† Per 100 g soil.

the *p*H will be lower the higher the cation exchange capacity-anion exchange capacity ratio. This reaction (*p*He) may vary between 2.6 to 5.4 (9, 10, 11). According to this procedure, the *p*H will not be below the *p*H of an H-soil or colloid. If the anion exchange capacity is measured below the *p*H of the H-colloid, there is a tendency of Al and Fe to go into solution with the resulting precipitation of aluminum and iron phosphates. Hence, if the reaction is below *p*He , a somewhat higher anion exchange capacity will be indicated.

The suggested procedure differs, therefore, from other methods where a constant *p*H was chosen, such as *p*H 4 in the Piper (14) and *p*H 5.7 in the Dean-Rubins (3) methods. According to Bass and Sieling (1) the Piper method yields far higher values than the proposed method. Thus,

the anion exchange capacities of Alamance, Cecil, and White Store soils were: 4.9, 13.3, and 11.1 meq. for the present method, and 22.5, 46.5, and 40.5 meq. for the Piper method. The Dean-Rubins method was used by Prabhu (15). With this method the C/A ratio of kaolinite, illite, and montmorillonite was 1.13, 1.08, and 2.64, respectively. Hence, a distinction between kaolinite and illite cannot be made. With the present proposed method, however, the C/A ratios of illite are higher than those of kaolinite (9).

CATION EXCHANGE CAPACITY-ANION EXCHANGE CAPACITY RATIOS (C/A) AND *p*He IN RELATION TO TYPE OF COLLOID

The C/A ratios and *p*He values have been proposed as a means of characterizing the predominant types of colloid in soil (2, 9, 10, 11). A summary of these data showing the C/A-*p*He relationships of different mineral colloids is presented graphically in Figure 1. The organic colloid was shown to have a high C/A ratio and low *p*He (9). This indicated a very low

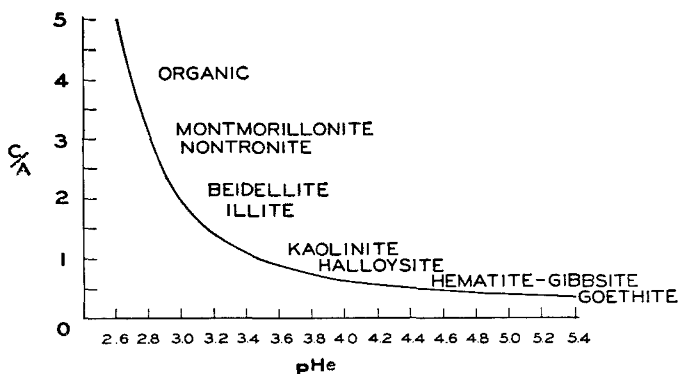

FIG. 1.—C/A=*p*He relationships of mineral colloids.

anion exchange capacity and a very high cation exchange capacity. In fact, when an organic soil colloid (peat) was digested with HCl, no detectable anion exchange was obtained. Hence, on natural soils anion exchange is due entirely to the presence of the clay minerals, including gibbsite, goethite, hematite, and the amorphoxide hydrates of Fe and Al. If the C/A ratio of a natural soil is sought, the organic matter must first be removed and the exchange properties again determined. An approximation of the C/A ratio of the mineral colloid may also be obtained by subtracting from the total C.E.C. that portion due to the organic matter. This is possible only where the C.E.C. of the organic colloids is known. A third possibility is that of interpolating C/A from *p*He values. This is due to the observation (2, 9) that *p*He is not as greatly influenced by

increases in C/A due to organic colloids as it is by increases due to mineral colloids. For this purpose the graph (Fig. 1) should serve as a guide.

Using the proposed procedure, a large number of soils have been studied (Table 1). It is to be noted that soils from the South and Southeast of the United States have lower C/A and higher *p*He values than those of the Middlewest or West. These differences in fundamental properties have a great influence upon problems of nutrient availability, liming, and fertilization. The characterization of soils by means of the proposed method should be useful, therefore, as an aid in the interpretation of some of these problems.

SUMMARY

A rapid method for the determination of cation and anion exchange properties and *p*He of soils is described.

For the determination of cation exchange properties, 2 to 8 g of soil (depending upon the cation exchange capacity) is weighed into special leaching tubes and 20 ml barium chloride-triethanolamine buffer (*p*H 8.1) is added. The sample is then leached with 20 ml barium chloride and the excess Ba removed by leaching with H_2O. The combined leachates are first titrated (by difference) for H with HCl. A sulfate-oxalate mixture is then added and the Ca determined in the presence of $BaSO_4$ by titrating with $KMnO_4$. In an alternate procedure the Ba is removed as the sulfate and Ca determined by means of a flame photometer. The Mg is determined colorimetrically with thiazol yellow and the K and Na are determined flame photometrically.

For the determination of cation exchange capacity, the Ba-soil is leached with 50 ml $CaCl_2$ and the Ba in the leachate measured colorimetrically following precipitation as $BaCrO_4$ or flame photometrically in the presence of $CaCl_2$.

The anion exchange capacity is determined on the Ca-soil, corresponding to 0.2 meq. cation exchange capacity, by treatment with 20 ml of 0.03 N H_3PO_4. The *p*He and the phosphate retained are measured. In a separate sample the exchangeable PO_4 is determined by means of HCl-NH_4F. The molybdo-vanadate phosphate method is employed.

Data relating to the characterization of soils from the standpoint of their C/A ratios and *p*He in relation to type of colloid are presented. Factors to be considered in the accurate determination of cation and anion exchange properties are discussed.

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THE SUGAR CONTENT OF HYDROL (CORN FEEDING MOLASSES)*

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The purpose of this paper is three-fold:

1. To give a brief description of the process by which hydrol is produced.
2. To present the composition of hydrol with particular reference to the multiple saccharide fraction.
3. To present a method for the determination of total sugars in hydrol and in products containing hydrol.

Hydrol, or corn sugar molasses, is obtained as a by-product in the manufacture of dextrose from starch. Annual production of hydrol in the United States is over 100,000 tons. It is used almost exclusively in sweetened livestock feed and in ensiled grass crops and is therefore of considerable interest to the Association of American Feed Control Officials and to the A.O.A.C.

Hydrol is obtained from the following process: a slurry of starch, water, and hydrochloric acid is introduced into pressure vessels at a solids content of 20 per cent and a *p*H of 1.5 . The hydrolysis is carried out under direct steam pressure (40 to 50 psi) for a period of 20 to 30 minutes. A solution with a dextrose content of approximately 87 per cent (ash-free, dry substance basis) is obtained. The remaining 13 per cent of carbo-

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hydrate material has either escaped complete hydrolysis or has been repolymerized from dextrose to higher sugars. The converter liquor is neutralized, refined, and concentrated to a solids content of 75 to 77 per cent. The heavy liquor is seeded with dextrose and crystallized to a solid phase of approximately 60 per cent and the crystalline dextrose is separated from the mother liquor in centrifugals. The removal of the dextrose approximately doubles the percentage of non-dextrose carbohydrates in the mother liquor. To improve the dextrose yield, this mother liquor is reconverted and again refined, concentrated, and crystallized, and yields a second crop of sugar crystals which are recycled to the virgin hydrolysate. The mother liquor from this second crystallization is *hydrol.* A complete description of the process of the manufacture of dextrose and hydrol from starch is given by Kerr (1).

An approximate analysis of commercial hydrol is shown in Table 1.

TABLE 1.—*Analysis of hydrol (dry substance basis)*[1]

Ash (sulfated)	10–12%
Sodium chloride	8.5–9.0%
Hydroxymethyl furfural[2]	1.6–2.0%
"Protein" (N×6.25)[3]	ca 0.3%
Total carbohydrate	86–88%
Reducing sugars (as dextrose)	73%
"True" dextrose	63%

[1] Dry substance 70–75%.
[2] Report of the "Hydrol Conference" (36th Industrial Conference), Northern Regional Research Laboratory, Peoria, Illinois, October 19, 1948.
[3] Largely protein decomposition products.

The composition of the carbohydrate portion of hydrol has been studied by Montgomery (2). Separation was accomplished by column chromatography using carbon-celite columns. U. S. Pat. No. 2,549,840 outlines the details of this procedure. The solution of mixed or unknown sugars is ion exchanged to remove ash and is then adsorbed on the carbon-celite column. After adsorption it is progressively eluted with water which removes the monosaccharides, then with 0.5 per cent aqueous phenol which removes disaccharides, and finally with 3.5 per cent aqueous phenol which removes oligosaccharides. A tentative analysis of the carbohydrate portion of a typical hydrol is shown in Table 2.

A method for the determination of total sugars in hydrol is needed by the A.O.A.C. and the Association of American Feed Control Officials. Current practice for the determination of total sugars in hydrol (or in sweetened livestock feed in which hydrol has been used) is to determine reducing sugars by one of the copper reduction methods and to calculate this as dextrose or invert sugar; then to invert at room temperature for determination of sucrose. The polysugars present in hydrol contain less than one reducing group per anhydro-glucose unit and thus are not quan-

TABLE 2.—*Tentative analysis of carbohydrate fraction of hydrol*[1]

		ASH-FREE PROTEIN-FREE MOISTURE-FREE BASIS
		per cent
Fraction displaced from carbon-celite column by elution with water alone		
Anhydrous dextrose isolated as dry hydrate, air-dried crystals		63
Anhydrous dextrose in residual sirup by n_D^{20}		4
Fraction displaced by 0.5% aqueous phenol (disaccharides)		
Gentiobiose, *approx.*	5	
Isogentiobiose, *approx.*	15	
Maltose, *approx.*	2	
Trehalose, *approx.*	1.5	
Other carbohydrates, *approx.*	1	23
Fraction displaced by 3.5% aqueous phenol		
Oligosaccharides larger than disaccharides (quantity estimated by n_D^{20} of sirup)		7.5
Total		97.5

[1] Separation by column chromatography, carbon-celite columns, Montgomery and Weakley, 1948. U. S. Northern Regional Research Laboratory, Peoria, Illinois.

titatively determined by a direct reducing power measurement. Neither are they hydrolyzed by inversion. Therefore, these methods do not determine the total sugars in hydrol. Examination of the structure of isogentiobiose (also called *isomaltose*, also *brachiose*), one of the disaccharides in hydrol, illustrates the inadequacy of the reducing sugar determination as

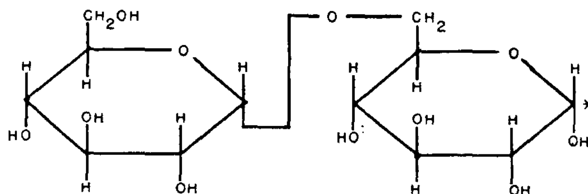

FIG. 1.—Structure of isogentiobiose (Haworth formulation). (★ Reducing end-group.)

an estimation of the total sugars in hydrol (Fig. 1). Isogentiobiose has two glucose units and only one available reducing group (terminal aldehyde group). Therefore a reducing sugar determination *calculated as dextrose* will account for only one-half of the isogentiobiose molecule.

With other multiple saccharides, one-half, one-third, or even less of the molecule will be accounted for, depending upon the degree of polymerization of the particular sugar.

Conversion of the multiple saccharides to dextrose by acid or enzyme hydrolysis was indicated; this could be followed by the determination of reducing sugars by one of the copper reduction methods. As the rate of enzyme hydrolysis is relatively slow, acid hydrolysis seemed more desirable. The principal object of this work was to determine the optimum conditions for the acid hydrolysis of the polysaccharides in hydrol with minimum loss of dextrose from decomposition or reversion to higher saccharides. Since the loss of some dextrose is unavoidable, a factor to correct for this loss was also developed.

This method of acid hydrolysis, followed by the determination of reducing sugars, is not only applicable to the determination of total sugars in hydrol, but may also be used for the determination of total sugars in sweetened feeds in which hydrol has been used. In the case of sweetened feed, the sugars are first extracted according to the A.O.A.C. method with hot 50 per cent alcohol (3), or simply by leaching with hot water. Either procedure may be followed by clarification with neutral lead acetate; in all cases the results are nearly the same.

PROCEDURE

1. *Extraction of sugars from sweetened livestock feed containing hydrol.*—According to *Methods of Analysis*, 7th Ed., **22.32**. Approximately 7.0 g (dry basis) of feed (volume estimated to be 5.0 ml) is extd with 50% aq. alcohol and clarified with neutral lead acetate as described. A 25 ml aliquot of the resulting 100 ml soln is dild to 250 ml and 50 ml is used for the detn of reducing sugars in the original ext. A 50 ml aliquot of the 100 ml lead acetate treated soln containing ca 1.0 g dry substance is used for hydrolysis.

Hot water extraction.—Approximately 100 ml of boiling H_2O is added to 6.0 g (dry basis) of feed. The mixt is stirred occasionally over a period of ½ hour. A small amount of filter aid is added and the mixt. transferred to a Büchner funnel. The residue is washed several times with hot H_2O. The filtrate is transferred to a 250 ml volumetric flask and dild to vol. (If needed, the soln may be clarified with neutral lead acetate.) A 100 ml aliquot of the 250 ml soln, containing ca 1.0 g dry substance, is used for hydrolysis. A 50 ml aliquot of the 250 ml soln is dild to 200 ml, and 50 ml is used for the detn of reducing sugars in the original ext.

2. *Acid hydrolysis.*—(Sulfuric acid and hydrochloric acid were considered. However, Pirt and Whelan (4) have shown that the rate of destruction of dextrose when heated with hydrochloric acid is ca two and one-half times greater than when heated with sulfuric acid. Our data confirm this work, and sulfuric acid is to be preferred.)

Method of hydrolysis.—The hydrolysis is carried out in a 1-liter flask equipped with a reflux condenser. Heat is provided by means of an electric heating mantle. The flask contg 1.0 g (dry substance) hydrol (or approximately 1.0 g of sugars extd from sweetened feed), 75 ml of 4 *N* sulfuric acid and 325 ml of water (resultant normality, 0.75) is heated to 100°C. in ca 25 min. and held at that temp. for 2½ hours. The hydrolysate is then cooled to room temp. as rapidly as possible in cold H_2O and neutralized to *p*H 4.5 to 6.0 with 50% NaOH. The liquor is transferred quantita-

tively to a 500 ml volumetric flask, made to vol. and filtered through a dry filter.

3. *Determination of reducing sugars.*—A Munson and Walker determination is made on a 50 ml aliquot of the filtrate as described in *Methods of Analysis*, 7th Ed. (5). The precipitated cuprous oxide is filtered, dissolved in ferric sulfate soln, and the ferrous sulfate produced is titrated with potassium permanganate. The end point, using ferrous phenanthroline indicator, is easily noted.

RESULTS

Hydrol was acid-hydrolyzed, using sulfuric acid and a dry substance of 0.25 per cent (1 g per 400 ml). The normality of the hydrolysate and the time of refluxing at 100° C were varied. Table 3 shows the conditions and the results (averages of duplicates). Maximum increase in per cent reducing sugars calculated as dextrose was obtained at 0.75 *N* and two and one-half hours. These data are also shown graphically in Figure 2.

TABLE 3.—*Acid hydrolysis of hydrol #311-150 A*[1]—*percentages of reducing sugars calculated as dextrose, dry basis*

	ORIGINAL HYDROL—72.5		
NORMALITY OF SULFURIC ACID	HYDROLYSIS AT 100°C.—HOURS		
	1	2½	4
1.5	85.7 84.5 86.0	84.7 84.7	82.8 82.7
0.75	85.5 85.5	{86.2 86.2 86.7 86.9}	85.1 85.1
0.35	81.8 82.1	85.4 85.8	85.6 85.6
0.10	77.2 76.4	79.5 79.7	81.5 82.0
0.05		77.4 77.3	

[1] Concentration of hydrolysate—0.25% dry substance.

Two runs in which the dry substance was increased to 1.00 per cent gave comparable results.

Lampitt, Fuller, and Goldenberg (7) have shown that when dextrose is heated with hydrochloric acid a loss in reducing power results. This effect was also studied by Pirt and Whelan (4) with both hydrochloric and sulfuric acids. We therefore subjected pure dextrose to the same conditions of hydrolysis which had given us the maximum per cent in-

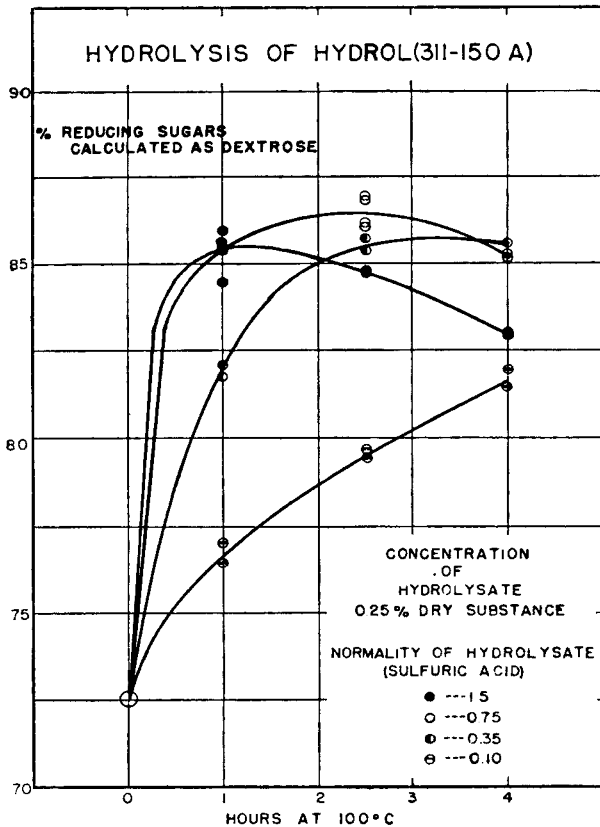

FIG. 2.—Effect of acid strength and time.

crease in reducing sugars for hydrol. Our results are shown in Table 4. It is noted that under these conditions, there is a loss of 1.4 per cent in the reducing power of dextrose. Therefore, to ascertain total dextrose, it is necessary to multiply the reducing sugar value after hydrolysis by a factor to correct for dextrose destruction or polymerization. Under our conditions of hydrolysis this factor is $(100+1.4)/100=1.014$, and this factor is used to correct for the loss which occurs when hydrolyzing hydrol or an extract of sweetened feed.

Dextrose, and dextrose plus 8.5 per cent sodium chloride, were also subjected to the A.O.A.C. (6) conditions of acid hydrolysis for starch. A loss in reducing power of approximately 3.5 per cent was obtained.

Hydrol, and sweetened feeds containing hydrol have customarily been analyzed for total sugars, calculated as either dextrose or invert, by a sim-

TABLE 4.—*Effect of hydrolytic conditions (for starch determination) on Bureau of Standards dextrose—100°C., 2.5 hours, acidity, and concentration as indicated*

SAMPLE	METHOD	ACID	NORMALITY	PER CENT DRY SUBSTANCE	PER CENT REDUCING SUGARS CALCULATED AS DEXTROSE		FACTOR FOR CORRECTION OF DEXTROSE LOSS
					BEFORE HYDROLYSIS	AFTER HYDROLYSIS	
N.B.S. dextrose	A.O.A.C. **22.34**	HCl	0.70	1.00	100.4	97.2	1.035
N.B.S. dextrose +8.5% NaCl	**22.34**	HCl	0.70	1.00	100.4	97.0	1.037
N.B.S. dextrose +8.5% NaCl	Proposed	H_2SO_4	0.75	0.25	100.4	99.0	1.014
					99.7	98.3	1.014
						98.3	1.014
					99.6	98.4	1.012
						98.2	1.014
							Av. 1.014

ple reducing sugar determination followed by a room temperature inversion to determine sucrose (*Methods of Analysis*, 7th Ed., **22.33**). Since the higher saccharides present in hydrol do not hydrolyze readily under these mild conditions, the results are very low. However, when these saccharides are subjected to hydrolytic conditions which would be essentially quantitative for starch, they do hydrolyze to dextrose. We have therefore used A.O.A.C. procedure **22.34** for starch to determine total sugars in hydrol. The iodine test, and solubility in 50 per cent alcohol, show that these carbohydrates are respectively neither starch itself nor dextrins. We have also hydrolyzed hydrol by our proposed milder hydrolytic technique in order to cause minimum loss of dextrose. These data are compared in Table 5.

The process for the manufacture of hydrol is such that one would expect virtually all of the carbohydrate to exist in the form of dextrose and its polymers. For this reason we have been of the opinion that all of the carbohydrate is sugar and that the total carbohydrate content should equal the total sugar content. We accordingly calculated the carbohydrate content by difference (subtracting non-carbohydrate solids) and compared this with the total sugars as determined by our method. The analyses of hydrol given in Table 6 show the close agreement between the value for total sugars obtained by difference and that found by our acid hydrolysis method followed by a Munson and Walker reducing sugar determination.

TABLE 5.—*Analysis of hydrol by A.O.A.C. vs. proposed method*

METHOD	ACID	NORMALITY	PER CENT DRY SUBSTANCE	PER CENT REDUCING SUGARS CALCULATED AS DEXTROSE, DRY BASIS	
				BEFORE HYDROLYSIS	AFTER HYDROLYSIS
A.O.A.C. 22.33*	HCl			72.5	73.4 74.7
A.O.A.C. 22.34 (for starch)	HCl	0.70	1.00	72.5	85.3 85.1
Proposed	H_2SO_4	0.75	0.25	72.5	86.2 86.2 86.7 86.9
Proposed	H_2SO_4	0.75	1.00	72.5	86.3 86.2

* *Methods of Analysis*, 7th Ed., 22.33, is room temperature inversion as for sucrose.

A correction for the destruction or repolymerization of dextrose and also another correction for the chemical gain occurring during hydrolysis has been made in order to obtain the true per cent total sugars in the original hydrol (see footnote, Table 6).

Total sugars by the acid hydrolysis procedure are seen to be about 18

TABLE 6.—*Analysis of hydrol*

	PER CENT DRY BASIS	
	HYDROL #311-150 A	HYDROL #337-60
Ash (sulfated)	10.5	11.7
"Protein" ($N \times 6.25$)[1]	0.2	0.2
Hydroxymethyl furfural	1.8	1.8
Total sugars (by difference)	87.5	86.3
Determination of total sugars by acid hydrolysis		
Reducing sugars calculated as dextrose		
Before hydrolysis	72.5	72.9
After hydrolysis ($\times$ 1.014)	87.7	87.4
Total sugars in original hydrol[2]	86.0	85.8
Per cent increase	18.5	17.8

[1] Largely protein-decomposition products.
[2] To obtain the % total sugars in the original hydrol we must subtract from the % reducing sugars calculated as dextrose (after hydrolysis) the chemical gain which occurred during hydrolysis.
Example—Hydrol #311-150 A
$87.7-0.111\,(87.7-72.5)=87.7-1.7=86.0$

per cent higher than the values obtained from the initial reducing power calculated as dextrose. Thus, for estimation purposes, multiplying the initial reducing sugars by a factor of 1.18 would serve as a fair approximation of the total sugars. In a series of four runs with different extraction methods, the per cent total sugars found in a sample of sweetened feed by the acid hydrolysis method showed values of from 17 to 20 per cent higher than the reducing sugars as dextrose found in the unhydrolyzed extract.

SUMMARY

A method for the determination of total sugars in hydrol and in sweetened feed containing hydrol has been developed. The hydrol, or extract of a sweetened feed after clarification, is subjected to an acid hydrolysis as for starch, to convert di- and higher saccharides to dextrose. This is followed by a determination of reducing sugars calculated as dextrose. A factor is given to correct for the loss of dextrose resulting from decomposition or reversion to higher saccharides under the conditions of hydrolysis. A correction must also be made for the chemical gain occurring during hydrolysis.

Two official A.O.A.C. procedures are used in tandem to make the analytical determinations. The essential new feature is the combination of these procedures. Certain refinements in these procedures are suggested but are of minor significance in comparison with the principle of acid hydrolysis of the multiple saccharides to determine "total sugars."

Where hydrol alone is involved a short-cut approximation of total sugars can be made by multiplying the initial reducing sugar content by the factor 1.18. This is probably accurate within ± 2.0 per cent and is recommended for routine feed control work.

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USE-DILUTION CONFIRMATION TESTS FOR RESULTS OBTAINED BY PHENOL COEFFICIENT METHODS*

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It has been commonly accepted that germicides used at dilutions equivalent in efficiency against *S. typhosa* to 5 per cent phenol at 20°C. in the phenol coefficient method will possess reasonable margins of safety for the destruction of infective agents likely to be the object of most general disinfection processes. The conventional method of arriving at the maximum safe use-dilution presumed to be equivalent in efficiency to 5 per cent phenol in the method is to multiply the phenol coefficient number found by the figure 20 to determine the number of parts of water in which 1 part of product is to be incorporated. While there may be considerable reason to doubt that dilutions of the various types of commercial germicides made up according to this formula do have germicidal efficiencies equivalent to 5 per cent phenol, this procedure has, with certain types of products in the past, provided for a reasonable margin of safety for disinfecting floors, walls, equipment, and facilities from which most extraneous organic matter had been removed. This has been pointed out by Varley and Reddish (1) and Reddish (2), and was confirmed many times in the laboratory of the Insecticide Division with commercial samples.

However, the cleaning processes of floors, walls, and certain equipment are often of a superficial nature and cannot be relied upon to reduce the amount of interfering organic matter or the number of bacteria to very low levels. During the last 10 years a rather alarming increase has been noted in the number of commercial products which, under these conditions, do not provide adequate margins of safety for disinfection even though they bear apparently valid phenol coefficient claims. It should be made a matter of record that most of these products will disinfect surfaces at the dilution indicated to be safe by the phenol coefficient number if they are applied after very thorough cleaning operations, but ordinary practices in janitorial services, home and farm sanitation programs, and even in hospital maintenance schedules cannot be relied upon to provide floor, wall, and fixed equipment surfaces of sufficient cleanliness to assure this result.

It appeared necessary, therefore, to develop some confirmatory test procedures which could be employed as a check on the practical significance of phenol coefficient values. Use-dilution testing of disinfectants was proposed by Mallman and Hanes (3) in 1944, and a procedure of this

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type was compared directly with the phenol coefficient method by Mallman and Leavitt (4) in 1948. As a result of these comparisons, it was reported that techniques of this kind provide a more dependable index to the actual safe use-dilution in the field than the phenol coefficient test, particularly with those products that exhibit high phenol coefficients.

The use-dilution method described by Mallman and Leavitt and numerous modifications of this method have been studied over a 5-year period in which many direct comparisons have been made on commercial germicides of all types. The results checked with those obtained in the phenol coefficient method and in tests conducted under conditions of actual use. In these studies particular attention has been given to the development of a procedure of sufficient precision to warrant acceptance for referee work, and to the accuracy of the end result in terms of actual disinfecting value.

Out of these studies, use-dilution methods have been developed for the specific purpose of confirming phenol coefficient values. As was found with the Mallman and Leavitt method, the results appear to provide more dependable indices to actual safe use-dilutions in the field than the phenol coefficient test, particularly with those products compounded from chemicals which are germicidally active at high dilutions. Also, collaborative test data have been obtained which indicate that they have sufficient precision to justify acceptance for referee purposes.

METHOD I

(Using *Salmonella cholerasuis*)

REAGENTS

(a) *Culture media.*—(1) *Nutrient broth.*—Boil 5 g beef extract (Difco), 5 g NaCl, and 10 g Armour peptone (quality specially prepared for disinfectant testing) in 1 l H_2O 20 min., adjust to pH 6.8 and make to vol. with H_2O. Filter thru paper, place 10 ml quantities in 20×150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lb pressure for 20 min.

(2) *Nutrient agar.*—Dissolve 1.5% Bacto agar (Difco) in nutrient broth and adjust to pH 7.2–7.4; place 15 ml quantities in 25×150 mm tubes, plug with cotton, sterilize at 15 lb pressure for 20 min., slant, and allow to solidify at room temp.

(3) *Subculture media.*—Use (a), (b), or (c), whichever gives lowest result:

(a) Nutrient broth described in (a)(1).

(b) *Fluid thioglycollate medium U.S.P. XIII.*—Mix 0.75 g l-cystine, 0.75 g agar, 2.5 g NaCl, 5.5 g dextrose, 5.0 g H_2O-sol. yeast ext., 15.0 g pancreatic digest of casein with 1 l H_2O; heat to dissolve on H_2O bath, add 0.5 g Na thioglycollate or 0.3 g thioglycollic acid, and adjust with N NaOH to pH 7.0±0.1; reheat without boiling and filter thru moistened filter paper; add 1.0 ml freshly prepd 0.1% Na resazurin soln; tube in 10 ml quantities in 20×150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lb steam pressure 20 min.; cool at once to 25° and store at 20–30°.

(c) *Letheen broth.*—Dissolve 0.7 g lecithin (azolectin) and 5.0 g sorbitan monooleate ("Tween 80") in 400 ml hot H_2O and boil until clear; add 600 ml aq. soln of 5.0 g beef extract (Difco), 10.0 g peptone (Armour), and 5 g NaCl, and boil 10 min.; adjust with N NaOH and/or N HCl to pH 7.0±0.2 and filter thru coarse

filter paper; tube in 10 ml quantities in 20×150 mm bacteriological test tubes, plug with cotton, and sterilize at 15 lb pressure 20 min.

With oxidizing products and products formulated with toxic compounds contg certain heavy metals like Hg, (b) will usually give the lowest result. With products contg cationic surface active materials, (c) will usually give lowest results.

(b) *Test organism, Salmonella cholerasuis* (A.T.C.C. 10708).—Carry stock culture on nutrient agar slants. Transfer once a month and incubate new stock transfer 2 days at 37°, then store at room temp. From stock culture inoculate tube of nutrient broth and incubate at 37°. Make 3 consecutive 24 hr transfers, then inoculate tubes of nutrient broth (2 for each 10 carriers to be tested) using one loop of inoculum with each tube, and incubate at 37° for 44–48 hrs.

(c) *Phenol.*—Use phenol, U.S.P., which has congealing point 40° or above. Use 5% soln as stock soln and keep in well-stoppered amber bottles in relatively cool place, protected from light. Standardize with 0.1 *N* K or Na bromide-bromate soln, **39.18.**

(d) *Sterile distilled water.*—Prep. stock supply distd H_2O in 1 l flasks, plug with cotton, sterilize at 15 lb pressure for 20 min. and use to prep. dilns of medicants.

(e) *Asparagine.*—Make stock supply of 0.1% soln of asparagine ("Bacto") in distd H_2O in Erlenmeyer flasks of convenient size, plug with cotton, and sterilize at 15 lb for 20 min. Use to cover metal carriers for sterilization and storage.

(f) *N* NaOH.—Maintain stock supply of NaOH soln of ca *N* (4%) for cleaning metal carriers prior to use.

APPARATUS

(a) *Glassware.*—1, 5, and 10 ml volumetric pipets; 1, 5, and 10 ml Mohr pipets graduated to 0.1 ml or less; 100 ml stoppered cylinders graduated in 1 ml divisions; Pyrex lipped test tubes 25×150 mm; straight side Pyrex test tubes 20×150 mm; 15×110 mm Petri dishes, 100 ml, 300 ml, and 1 l Erlenmeyer flasks. Plug all tubes and flasks with cotton. Sterilize all glassware 2 hrs in hot air oven at 180° employing closed metal containers for pipets and Petri dishes.

(b) *Water bath.*—Insulated relatively deep H_2O bath with cover having at least 10 well spaced holes which admit medicant tubes but not their lips.

(c) *Racks.*—Any convenient style. Conventional wire racks or blocks of wood with deep holes are satisfactory. Have holes well placed to insure quick manipulation of tubes.

(d) *Transfer loops and needles.*—**(1)** Make 4 mm (inside diam.) single loop at end at 2–3 inch Pt alloy wire No. 23 B&S gauge. Have other end in suitable holder (glass or Al rod). Bend loop at a 30° angle.

(2) Make 3 mm right angle bend at end of 2–3 inch nichrome wire No. 18 B&S gauge. Have other end in suitable holder (glass or Al rod).

(e) *Carriers.*—Polished stainless steel cylinders (penicillin cups)* with an outside diam. of 8±0.1 mm.†

(f) *Petri dishes.*—Have ca 6 sterile Petri dishes matted with a layer of S&S No. 597, 9 cm filter papers.

DETERMINATION

Soak ring carriers overnight in *N* NaOH soln, rinse with tap H_2O until rinse H_2O gives neutral reaction to phenolphthalein, then rinse 2 times with distd H_2O; place cleaned ring carriers in multiples of 10 in cotton plugged Erlenmeyer flasks or 25×150 mm cotton plugged Pyrex test tubes, cover with 0.1% soln of asparagine **(e)**, sterilize at 15 lb for 20 min., cool and hold at room temp. Transfer 20 sterile ring carriers using flamed nichrome wire hook into 20 ml of a 44–48 hr nutrient broth

* See Federal Register, Vol. 12, No. 67, p. 2217, April 4, 1947.
† (May be purchased from Erickson Screw Machine Products Co., 25 Lafayette Street, Brooklyn, New York.)

test culture in a sterile 25×150 mm medicant tube. After 15 min. contact period, remove cylinders, using flamed nichrome wire hook, and place on end in vertical position in a sterile Petri dish matted with filter paper. Place in incubator at 37° and allow to dry for no less than 10 min. and no more than 60 min. Hold the broth culture for detn of its resistance to phenol by the phenol coefficient method.

From the 5% stock soln make 1–90 and 1–100 dilns of the phenol directly into medicant tubes. Place tube for each diln in H_2O bath and allow to come to temp. Make stock soln of the germicide to be tested in sterile glass stoppered cylinder. From this soln make 10 ml dilns to be tested depending upon the phenol coefficient found and/or claimed against *S. typhosa* at 20° directly into each of ten 25×150 mm medicant tubes, and then place the 10 tubes in the H_2O bath at 20° and allow to come to temp. Det. the diln to be tested by multiplying the phenol coefficient number found and/or claimed by 20 to det. the number of parts of H_2O in which one part of germicide is to be incorporated.

Add 0.5 ml of the test culture suspension to the 1–90 diln of the phenol control; after a 30 sec. interval, add 0.5 ml to the 1–100 diln of the control, using sterile cotton plugged pipets. After adding culture, agitate tubes gently but thoroly to insure even distribution of bacteria and replace in bath; 5 min. after seeding first medicant tube, transfer 1 loopful of mixt. of culture and dild phenol from medicant tube to corresponding subculture tube. At end of 30 sec. interval, transfer loopful from second medicant tube; 5 min. after making first set of transfers begin second set of transfers for 10 min. period; and finally repeat for 15 min. period. Use technique of loop sampling, flaming loop and mouths of tubes and agitating medicant and subculture tubes as outlined in phenol coefficient method. Incubate subcultures at 37° for 48 hrs and read results. Resistance in the 44–48 hr culture of *S. cholerasuis* should fall within range specified for the 24 hr culture of *S. typhosa* in the phenol coefficient method.

Add one contaminated dried cylinder carrier to each of the 10 tubes of the use-diln of the germicide to be tested at 1 min. intervals. Thus, by the time the 10 tubes have been seeded, 9 min. will have elapsed plus a 1 min. interval before transfer of the first carrier in series to an individual tube of subculture broth. This interval is a constant for each tube with the prescribed exposure period of 10 min. The 1 min. interval between transfers allows adequate time for flaming and cooling nichrome wire hook and making transfer in a manner so as to drain all excess medicant from carrier. Flame lips of medicant and subculture tubes in conventional manner. Immediately after placing carrier in the medicant tube, swirl tube 3 times before placing it back into bath. Shake subculture tubes thoroly, incubate 48 hrs at 37°, and report results as + (growth) or − (no growth) values. Where there is reason to suspect that lack of growth at the conclusion of incubation period may be due to bacteriostatic action of medicant adsorbed on carrier which has not been neutralized by subculture medium employed, each ring shall be transferred to a new tube of sterile medium and reincubated for an addnl period of 48 hrs at 37°. Results showing no growth on all 10 carriers would confirm the phenol coefficient number found. Results showing growth on any of the 10 carriers should be considered as indicating the phenol coefficient number to be an unsafe guide to the diln for use. In the latter case, the test should be repeated using lower dilns of the germicide under study. The maximum diln of the germicide which kills the test organism on the 10 carriers in the 10 min. interval would represent the maximum safe use-diln.

METHOD II

(Using *Micrococcus pyogenes* var. *aureus*)

Proceed as directed in Method I except to change phenol dilns and test organism. Use culture of *M. pyogenes* var. *aureus* F.D.A. 209, A.T.C.C. No. 6538 having at

least the resistance specified for the 24 hr culture at 20° in the phenol coefficient method.

If the germicide does not kill the test organism on 10 of the 10 carriers at the dilution indicated to be safe by the *S. typhosa* coefficient found and/or claimed, then it should not be recommended at this dilution for disinfecting in hospitals or places where pyogenic bacteria are likely to have special significance.

Results secured on selected samples of commercial germicides of different types when using the methods outlined are presented in Table 1.

In selecting the germicides listed in Table 1, primary consideration was given to securing examples of the variety of results which may be expected with the different types. The ones selected should not be considered as representative of all available germicides of the types listed.

With the 5 pine oil disinfectants having phenol coefficients ranging from 3.0 to 5.0, the maximum safe use-dilutions indicated by the coefficients claimed were confirmed by the use-dilution test when *S. cholerasuis* was employed. When the use-dilution test was employed using *M. pyogenes* var. *aureus*, two of these killed the organism at a dilution of 1:5, and the other three killed the organism when applied undiluted. While these results indicate that this class of products would not be effective against pyogenic bacteria when used at any practical dilution, they were completely out of line with previous experiences with pine oil disinfectants. In the past, products of this class have not been found effective against pyogenic bacteria at any dilution. Further study showed that these products also killed *M. pyogenes* var. *aureus* when used undiluted or at a dilution of 1:5 in the phenol coefficient procedure, although not at higher dilutions. This characteristic was apparently due to the use of synthetic anionic detergents in the emulsifiers employed which were germicidal at low dilutions for gram positive organisms. This activity does not seem to be great enough to have any practical significance, but seems to be characteristic of many pine oil disinfectants currently being produced. This effect will be investigated further.

With the 5 pine odor germicides, the results are considerably more variable. This might be expected from the more heterogenic character of the formulas employed with this class of materials. With germicide 1, a phenol coefficient of 5.0 was claimed and found. However, the highest dilution found to be effective in the use-dilution confirmation tests was 1:5. This would seem to indicate that the phenol coefficient claim of 5.0 was misleading. With germicide 2, a phenol coefficient of 3.0 was claimed and found. The maximum safe use-dilution of 1:60 indicated by this value was confirmed in the use-dilution test using *S. cholerasuis*. However, it was not effective against pyogenic bacteria even when tested undiluted in the use-dilution test using *M. pyogenes* var. *aureus*. A similar

TABLE 1.—*Results obtained with selected commercial germicides of different types in the phenol coefficient and use-dilution confirmation tests*

GERMICIDES BY TYPE	PHENOL COEFFICIENT (*S. typhosa* at 20°C.)		USE-DILUTION CONFIRMATION TESTS*	
	CLAIMED	FOUND	MAX. SAFE USE DILUTION (*S. cholerasuis*)	MAX. SAFE USE DILUTION (*M. pyogenes var. aureus*)
(Pine Oil)				
1	5.0	5.0	1:100	Undiluted
2	3.0	3.0	1:60	1:5
3	5.0	5.1	1:100	Undiluted
4	5.0	4.7	1:100	1:5
5	4.0	4.2	1:80	Undiluted
(Pine Odor)				
1	5.0	5.0	1:5	1:5
2	3.0	3.0	1:60	None
3	5.0	5.0	1:100	1:5
4	5.0	5.0	1:100	None
5	5.0	5.0	1:20	1:100
(Phenol Emulsifying Type)				
1	5.0	5.2	1:40	1:20
2	5.0	5.0	1:100	1:20
3	5.0	4.7	1:80	1:20
4	5.0	5.2	1:60	1:5
5	5.0	5.0	1:40	1:80
6	32.0	36.0	1:640	1:640
(Phenolic Soluble Type)				
1	4.0	4.0	1:80	1:40
2	4.0	4.2	1:80	1:80
3	5.0	6.7	1:100	1:80
4	4.0	4.4	1:80	1:80
(Quaternary Ammonium)				
1	25.0	25.0	1:100	1:100
2	20.0	22.2	1:50	1:50
3	25.0	23.3	None	None
4	5.0	5.0	1:5	1:5
5	25.0	25.6	1:60	1:60

* The maximum dilutions tested were no greater than those indicated to be safe by conventional calculations using the phenol coefficients claimed.

result was secured with germicide 4 of this series where a phenol coefficient of 5.0 was claimed and found. With germicide 3, where a phenol coefficient of 5.0 was also claimed and found, confirmation was secured in the use-dilution test with *S. cholerasuis*, but the maximum safe use-dilution with *M. pyogenes* var. *aureus* was only 1:5. Germicide 5 of this group possessed the phenol coefficient of 5.0 claimed for *S. typhosa*, but in the use-dilution test, a 1:20 dilution was the maximum which would kill *S. cholerasuis*

on 10 out of 10 carriers. On the other hand, this product was effective in the use-dilution test, at the dilution indicated to be safe, when *M. pyogenes* var. *aureus* was employed. This result is the reverse of what might normally be expected, but seems to have been due to the presence of phenols specific for Gram positive bacteria. With the 6 phenolic disinfectants of the emulsifying type, only samples 2 and 6 gave results in the use-dilution test with *S. cholerasuis* which confirmed the phenol coefficient claims. With sample 1, a phenol coefficient of 5.0 was claimed and a value of 5.2 was found. However, a dilution of 1:40 was necessary to kill *S. cholerasuis* in the confirmation test. With samples 3, 4, and 5, coefficients of 5.0 were also claimed against *S. typhosa*. Values of 4.7, 5.2, and 5.0 were found, respectively. Nevertheless, none of the three would kill *S. cholerasuis* in the use-dilution method at the indicated safe use-dilution of 1:100; dilutions of 1:80, 1:60, and 1:40 were necessary to secure this result. Sample 6 contained a high percentage of high boiling cresols, but was essentially an emulsion type product. Only one of the 6 products in this class killed *M. pyogenes* var. *aureus* in the use-dilution test at the dilution indicated to be safe by the *S. typhosa* coefficient. With sample 5, *M. pyogenes* var. *aureus* was killed at a higher dilution than *S. cholerasuis*. This was apparently due to the presence of synthetic phenols specific for Gram positive organisms. With sample 1, a use-solution twice as concentrated as that necessary with *S. cholerasuis* was required when *M. pyogenes* var. *aureus* was used. With sample 2, a use-solution 5 times as concentrated was necessary; with sample 3, a use-solution 4 times as concentrated was required, and with sample 4, a solution 12 times as concentrated was necessary. The failure to kill *M. pyogenes* var. *aureus* at the dilutions indicated to be safe by the *S. typhosa* coefficient, or the use-dilution test using *S. cholerasuis*, cannot be considered as unusual in the light of the reports of Brewer and Ruehle (5) and Klarmann and Shternov (6) which point out the weakness of the *S. typhosa* coefficient for determining the effectiveness of products of this class as disinfectants for pyogenic bacteria.

With each of the 4 phenolic disinfectants of the soluble type, *S. cholerasuis* was killed in the use-dilution test at the dilution indicated to be safe by the phenol coefficient claimed for *S. typhosa*. With samples 2 and 4, this dilution was also found to be effective when *M. pyogenes* var. *aureus* was employed in the confirmation test. With samples 1 and 3, higher concentrations were necesssary to kill *M. pyogenes* var. *aureus* than were required for *S. cholerasuis*.

No germicides of the quaternary ammonium type have been found which will kill either *S. cholerasuis* or *M. pyogenes* var. *aureus* in the confirmation tests at the dilutions indicated to be safe by the *S. typhosa* coefficients claimed and found. With germicide 1, a dilution of 1:100 was found to be necessary to kill both organisms in the confirmation methods, although a dilution of 1:500 was indicated to be safe by the phenol co-

efficient claimed and found. Similarly germicide 2 was found to require a 1:50 dilution to kill both organisms in the confirmatory methods, although a dilution of 1:400 was indicated by the *S. typhosa* coefficient claimed and found. With germicide 3, tests showed that the product would not disinfect in the confirmatory method with either organism, even when used undiluted, although definite phenol coefficients values could be secured. The results with germicides 4 and 5 were similar to those found with germicides 1 and 2 of this group.

TABLE 2.—*Comparison of results on common phenolic germicides by the phenol coefficient test and the use-dilution confirmation test. Test organism*—M. pyogenes *var.* aureus

GERMICIDE	PHENOL COEFFICIENT FOUND	HIGHEST 10 MIN. KILLING DILN. IN PHENOL COEFF. METHOD	HIGHEST 10 MIN. KILLING DILN. IN USE-DILN. CONFIRMATION METHOD	POSSIBLE SAFE USE-DILN.*
1	3.7	1:220	1:80	1:74
2	3.2	1:200	1:80	1:64
3	3.0	1:180	1:80	1:60
4	3.0	1:180	1:80	1:60
5	2.5	1:150	1:60	1:50
6	1.6	1:110	1:40	1:32
7	1.0	1:60	1:30	1:20
8	0.3	1:18	1:5	1:6

* Determined by multiplying phenol coeff. number by 20.

In Table 2, results by the phenol coefficient method on selected phenolic disinfectants of both the emulsifiable and soluble types are compared with those by the use-dilution confirmation method. *M. pyogenes* var. *aureus* was used as the test organism.

The data in Table 2 show clearly that a much lower dilution is required to disinfect carriers contaminated with *M. pyogenes* var. *aureus* in the use-dilution confirmation test than is required to kill this test organism in the phenol coefficient procedure. However, the critical killing dilution in the method does appear to be slightly higher than a theoretically safe use-dilution determined by multiplying the *M. pyogenes* var. *aureus* coefficient found by the number 20 to determine the number of parts of water in which one part of germicide should be incorporated.

While a variety of actual use tests have been conducted to determine the relative efficacies of the phenol coefficient and use-dilution confirmation tests as indices to practical disinfecting values, only one test conducted on floors and one test conducted on surgical instruments will be reported herein by way of illustration.

In the use tests on floors, the following procedure was employed: One hundred grams of chicken feces were mascerated in 100 ml of a 48 hr broth culture of *S.*

cholerasuis and then added with vigorous stirring to 10 quarts of water in an enamel pail. This water was then employed to mop up a ceramic tile floor in a room of approximately 10 by 15 feet. After drying, the floor was visually clean of excess organic matter, but was at the same time heavily contaminated with bacteria and soluble organic matter. The individual tiles in the floor were approximately 6 inches square. Using sterile cotton swabs, individual tiles were thoroughly wet with various dilutions of the selected germicides. At the conclusion of a 10 min. interval, each treated tile and untreated control tiles were swabbed with standardized sterile dry cotton swabs, and these were immediately taken into the laboratory for bacteriological analysis. This consisted of transferring the cotton swab into a 100 ml. sterile dilution blank containing a 5% aqueous solution of Tamol N and shaking thoroughly. From this initial suspension, dilution plate counts using nutrient agar were made. Also, 1 ml aliquots were used to inoculate tubes of lactose broth (which were incubated for presumptive evidence of the presence of coliform bacteria) and Bacto tetrathionate broth to recover surviving cells of *Salmonella*. All agar plates were incubated at 37°C. for 48 hrs. and counts were made using a Quebec colony counter. The lactose broth tubes were incubated at 37°C. for 48 hrs, after which time the growth in all tubes showing gas production was streaked on E.M.B. agar plates for incubation and identification of *E. coli* colonies. The tetrathionate broth tubes were incubated for 24 hrs. at 37°C., and then streaked out on Bacto SS agar plates for the isolation of colonies of *Salmonella*.

The results secured in this study are given in Table 3.

The data in Table 3 clearly indicate that the maximum safe use-dilution found in the use-dilution confirmation test using *S. cholerasuis* will provide for the disinfection of floors even when the maximum safe use-dilution indicated by the conventional method of interpreting the phenol coefficient found does not. With phenolic disinfectant 1, a phenol coefficient of 4.0 was claimed and found. This indicated that the maximum safe use-dilution to disinfect would be 1:80. This dilution was found to be adequate in the use-dilution confirmation test using *S. cholerasuis*. When tested on the floor, it reduced the total bacterial count of the floor by 99.918 per cent and eliminated the coliform and *Salmonella* organisms known to be present. On the other hand, phenolic disinfectants 2 and 3 which had phenol coefficients of 5.0 did not kill at the expected safe use-dilution of 1:100 in the use-dilution confirmation test using *S. cholerasuis*. They did kill at dilutions of 1:40 and 1:60, respectively, in this test. When tested on the floor at a dilution of 1:100, neither product eliminated the coliform and *Salmonella* organisms known to be present, although they did reduce the total bacterial counts by 93.299 and 98.609 per cent, respectively. At the dilutions of 1:40 and 1:60 indicated to be safe by the use-dilution confirmation test using *S. cholerasuis*, the total bacterial counts were reduced by 99.755 and 99.745 per cent and all coliform and *Salmonella* organisms were killed. With the Pine-Odor Quaternary Ammonium preparation, a safe use-dilution of 1:100 was indicated by the phenol coefficient claimed and found. This product did not kill in the use-dilution confirmation test using *S. cholerasuis* at a dilution of 1:100, but did kill in this test as a dilution of 1:5. When tested on the floor at

TABLE 3.—*Correlation of results obtained in phenol coefficient and use-dilution confirmation methods with results of floor disinfecting studies*

GERMICIDE	MAXIMUM SAFE USE-DILN. INDICATED BY:		DILUTIONS TESTED ON FLOOR	NUTRIENT AGAR PLATE COUNTS		BACTERIOLOGICAL CONFIRMATION TESTS FOR	
	S. typhosa COEFFICIENT	CONFIRMATION TEST USING *S. cholerasius*		AVERAGE COUNT PER 6 SQUARE TILE SWAB	PER CENT REDUCTION OVER CONTROL	*Coli*	*Salmonella*
Untreated control	—	—	—	98,599,000	0	+	+
Phenolic disinfectant 1	1–80	1–80	1–80	80,000	99.918	–	–
Phenolic disinfectant 2	1–100	1–40	1–100 1–40	5,600,000 140,000	93.299 99.755	+ –	+ –
Phenolic disinfectant 3	1–100	1–60	1–100 1–60	1,370,000 150,000	98.609 99 745	+ –	+ –
Pine Odor quaternary ammonium prepn	1–100	1–5	1–100 1–5	560,000 10,000	99.431 99.989	+ –	+ –
Pine Oil disinfectant	1–100	1–100	1–100	140,000	99.755	–	–
Phenol	1–20	—	1–20	20,000	99.979	–	–

dilutions of 1:100 and 1:5, the total bacterial counts were reduced by 99.431 and 99.989 per cent respectively, but at the 1:100 dilution the coliform and *Salmonella* organisms known to be present were not eliminated. They were eliminated at the 1:5 dilution. The pine oil disinfectant which was found to have a phenol coefficient of 5.0 killed *S. cholerasuis* at a dilution of 1:100 in the use-dilution confirmation method. When tested on the floor it reduced the bacterial count by 99.755 per cent and eliminated both the coliform bacteria and *Salmonella*. A 1:20 dilution of phenol which was tested on the floor as a control reduced the total bacterial count by 99.979 per cent and eliminated all coliform bacteria and *Salmonella*.

In the study on surgical instruments, 5 typical pyogenic bacteria were used; namely, *Streptococcus pyogenes, Streptococcus fecalis, Streptococcus agalaeticae, Micrococcus pyogenes* var. *albus*, and *Micrococcus pyogenes* var. *aureus*. These were grown in a 50–50 mixture of soy broth and whole blood. Twenty-four hour cultures, incubated in this medium at 37.5°C., were employed for contaminating heat sterilized detachable scalpel blades. All blades were drained and dried for 10 minutes before exposure for 10 minutes at 20°C. to selected dilutions of a phenolic disinfectant of

the emulsifiable type. It had been determined that the phenolic germicide used in this study possessed, according to the *S. typhosa* phenol coefficient claimed and found, a possible safe use-dilution of 1:100; a maximum killing dilution against *S. cholerasuis* in the use-dilution confirmation test of 1:60; a maximum killing dilution against *M. pyogenes* var. *aureus* in the phenol coefficient method of 1:20; and a maximum killing dilution against *M. pyogenes* var. *aureus* in the use-dilution confirmation test of 1:5. These four dilutions were employed in the study.

Ten contaminated blades were exposed to each dilution for 10 minutes with each test organism. Subcultures were made in soy broth with 0.1 per cent added whole blood and were incubated for 48 hours at 37°C. All blades exposed to the 1:5 dilution were subcultured in fresh tubes of media and incubated for an additional period of 48 hours, since sufficient medicant was carried over in the first transfer to cause the formation of cloudy precipitates in the first subculture tubes.

The results are reported in Table 4.

TABLE 4.—*Results of tests on a phenolic disinfectant of the emulsifiable type using detachable scalpel blades contaminated with a blood film carrying various pyogenic bacteria*

	POSSIBLE SAFE USE-DILUTION TESTED AS INDICATED BY:							
	S. typhosa COEFF.		*S. cholerasius* CONFIRMATION TEST		*M. pyogenes* VAR. *aureus* 10 MIN. KILLING DILN. COEFF. PROCEDURE		*M. pyogenes* VAR. *aureus* CONFIRMATION TEST	
DILUTION	1:100		1:60		1:20		1:5	
ORGANISM	NUMBER BLADES TESTED	NUMBER BLADES +*	NUMBER BLADES TESTED	NUMBER BLADES +	NUMBER BLADES TESTED	NUMBER BLADES +	NUMBER BLADES TESTED	NUMBER BLADES +
Strep. pyogenes	10	10	10	10	10	0	10	0
Strep. fecalis	10	10	10	9	10	0	10	0
Strep. agalacticae	10	10	10	2	10	0	10	0
M. pyogenes var. *albus*	10	10	10	10	10	10	10	0
M. pyogenes var. *aureus*	10	10	10	10	10	10	10	0

* Indicates growth in subculture medium.

The data in Table 4 show clearly that the dilution of 1:100 indicated to be safe by the *S. typhosa* coefficient claimed and found would not disinfect surgical blades contaminated with a blood film in the presence of any of the five pyogenic organisms used. The dilution of 1:60 found to be effective against *S. cholerasuis* in the use-dilution confirmation test was also ineffective against all five pyogenic bacteria. The effective killing dilution of 1:20 found in the phenol coefficient procedure using *M. pyogenes* var. *aureus* was effective in disinfecting the blades when the three streptococci were employed. It was not effective in the case of the two staphytococci. All blades were disinfected at the dilution of 1:5 indicated to be safe in the use-dilution confirmation test with *M. pyogenes* var. *aureus*.

The procedures outlined were checked for precision in collaborative investigations in which two Federal laboratories, one State laboratory, two commercial testing laboratories, and two manufacturers' laboratories participated. Two unknown phenolic type disinfectants were employed. Sample 1 was of the soluble type and sample 2 of the emulsifiable type. The results have been summarized in Table 5.

Table 5 shows excellent agreement between the results of collaborators 1, 2, 3, 4, 5, and 6. Collaborator 7 found no end point with either germicide in any of the tests. The reason for this is not clear, but it would seem to be the inability to maintain the test cultures at the desired resistance levels to phenol. (The culture of *Salmonella cholerasuis* used was more resistant than specified in the procedure outlined and the culture of *M. pyogenes* var. *aureus* considerably less resistant than prescribed.)

A phenol coefficient of 4.0 had been claimed and found for unknown germicide 1. All seven laboratories found that this product killed *Salmonella cholerasuis* in the use-dilution confirmation test at the dilution of 1:80 indicated to be safe by this value (4 times 20). Six of the seven laboratories found that this product would also kill *M. pyogenes* var. *aureus* at a dilution of 1:80 in the use-dilution confirmation test. One found that a 1:60 dilution was necessary to secure this result.

With germicide 2, a phenol coefficient of 5.0 was claimed and found. Only 2 of the 7 collaborators found that the indicated safe use-dilution of 1:100 would kill *Salmonella cholerasuis* in the use-dilution confirmation test. The other 5 laboratories agreed that a 1:60 dilution was necessary to secure this result. Only one laboratory found that the dilution of 1:100 would kill *M. pyogenes* var. *aureus* in the use-dilution confirmation method. One found that a dilution of 1:10 was necessary for disinfection in this method, 3 found that a dilution of 1:5 was required, and 2 found that disinfection was not secured even at a dilution of 1:5.

DISCUSSION

These studies show that the described use-dilution confirmation tests can be applied, along with the existing phenol coefficient procedures, to provide a more accurate index than phenol coefficients alone to the actual value of chemical germicides for disinfecting articles, surfaces, and places where prior cleaning cannot be depended upon to remove all interfering organic matter or to reduce bacterial loads to low levels. The collaborative data reported clearly indicate that the procedures have sufficient precision for use in referee work.

The employment of multiple ring carriers at each dilution to be tested makes the procedures too cumbersome for most initial evaluations. However, ten carriers are necessary for the final determination of 100 per cent kill end points with many of the newer types of germicides which are active in very high dilution. The single and admittedly arbitrary time

Table 5.—*Results of collaborative studies on the use-dilution confirmation methods with two unknown phenolic disinfectants*

Unknown phenolic germicides	Dilution	Collaborating laboratories													
		1		2		3		4		5		6		7	
		Number rings tested	Number rings +	Number rings tested	Number rings +	Number rings tested	Number rings +	Number rings tested	Number rings +	Number rings tested	Number rings +	Number rings tested	Number rings +	Number rings tested	Number rings +
(Method I. Using *Salmonella cholerasuis*)															
1 (Soluble type)	1:80	9	0	10	0	10	0	10	0	10	0[1]	10	0	10	0
	1:60	10	0	10	0	10	0	10	0	10	0	10	0	10	0
2 (Emulsion type)	1:100	10	9	10	6	10	3	10	4	10	0[4]	10	4	10	0
	1:80	10	3	10	3	10	2	10	1	—	—	10	1	10	0
	1:60	10	0	10	0	10	0	10	0	—	—	10	0	10	0
Phenol Resistance Test Cultures		1:90		1:90		1:95		1:80		1:85		1:90		1:90	
(Method II. Using *M. pyogenes* var. *aureus*)															
1 (Soluble type)	1:80	9	0	10	5	10	0	10	0	10	0[3]	10	0	10	0
	1:60	10	0	10	0	10	0	10	0	10	0	10	0	10	0
2 (Emulsion type)	1:100	10	10	8	8	10	10	10	10	—	—	—	—	10	0
	1:80	10	10	—	—	10	10	10	10	—	—	—	—	—	—
	1:60	10	10	8	8	10	10	10	10	—	—	10	10	10	0
	1:20	10	9	10	10	10	9	10	4	10	10	10	4	10	0
	1:10	—	—	—	—	—	—	10	0	—	—	—	—	—	—
	1:5	10	0	10	10[2]	10	0	—	—	10	3	10	0	10	0
Phenol Resistance Test Cultures		1:60		1:60		1:60		1:60		1:65		1:65		1:80	

[1] Collaborator 5 reported no + rings at dilutions indicated and some − rings at all dilutions up to 1-500.
[2] Collaborator 2 reported that some negative results at dilution of 1:20 and 1:5 were found but were traced by subculture to the bacteriostatic effect of small amounts of the germicide carried into the subculture tubes.
[3] Collaborator 5 reported no + rings at a dilution of 1-100 but 9 + rings at 1:120.
[4] Collaborator 5 reported 6 + rings at a dilution of 1:120.

interval of 10 minutes is longer than the contact period which would be encountered in some applications, but represents about the average time that activity could be expected on floors, walls, and fixed equipment after mopping or spraying, since drying is usually quite complete within this time interval.

From the illustrations given it is apparent that, with some products, use of the phenol coefficient values according to the conventional method of calculating safe use-dilutions may not provide solutions which can be depended upon to disinfect. Where this is true, the phenol coefficient number can be misleading in that it provides an erroneous index to the true disinfecting value of the product. Consideration should be given, therefore, to prohibiting the use in labeling of coefficient numbers higher than those that can be confirmed by the use-dilution confirmation tests described.

SUMMARY

Two use-dilution test procedures for disinfectants have been described. One employs *Salmonella cholerasuis* as the test organism, the other *Micrococcus pyogenes* var. *aureus*.

Results secured by these procedures have been compared with results secured in the official phenol coefficient methods and in actual use tests on floors and surgical instruments. These comparisons indicate clearly that the use-dilution procedures described can be applied in supplementing phenol coefficient data to provide more accurate evaluations as to the safe use-dilutions of chemical germicides.

Both of these use-dilution tests have been evaluated collaboratively by seven laboratories and the results reported have been analyzed. These results indicate that the tests have sufficient precision to warrant use for referee purposes.

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THE RESISTANCE OF BACTERIAL SPORES TO CONSTANT BOILING HYDROCHLORIC ACID*

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Great variation is known to exist in the resistance of the endospores found in strains of individual *Bacilli* and *Clostridia*. For a proper evaluation of chemicals represented as sporocidal it is therefore essential to employ test culture spores carrying some predetermined resistance to a known chemical. Hydrochloric acid has been shown to be sporocidal and it has been the policy of the Insecticide Division to use this chemical as a standard for determining the resistance of spores used in tests of the sporocidal activity of commercial germicides.

Possibly the most common method for the standardization of hydrochloric acid is the constant boiling method. The procedure is outlined in most textbooks on quantitative analysis such as Kolthoff and Sandell (1) and in the *Official Methods of Analysis* (2). Thus, constant boiling hydrochloric acid can be considered as both a convenient and commonly recognized chemical standard. The exact hydrochloric acid concentration may vary slightly, depending on the atmospheric pressure. At 780 mm Hg the concentration will be 20.173 per cent and at 730 mm it will be 20.293 per cent or a mean molarity of approximately 5.5. The acid prepared and used in these studies had a concentration of 20.210 per cent.

In private communications, some bacteriologists have claimed that they could not obtain bacterial spores which would resist constant boiling hydrochloric acid solutions for measurable periods of time at 20°C. Also, it has been observed in the laboratory of this Division that many stock cultures of *Bacilli* and *Clostridia* do not produce spores that will withstand boiling hydrochloric acid at 20°C. On the other hand, it has been found that cultures of almost any species of these two genera can eventually be induced to produce spores which, when dried, will withstand this solution for 5 minutes. Many species can be induced to produce spores which will withstand this treatment for 30 minutes or longer. The various procedures employed in the Division's laboratories in obtaining resistant spores for testing purposes, exposing them to the hydrochloric acid standard as well as the unknown germicides, and subculturing to determine death of the spores, have been carefully evaluated and a preferred

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procedure developed. This procedure has been designed to reduce the hazard in handling dried spores of such pathogenic species as *B. anthracis* and *Cl. tetani* to a minimum, and to provide the technician with a constant supply of dried spores of known resistance to the control chemical for use in evaluating unknown sporocides.

METHOD

All test cultures are grown in liquid media. All broth media are made up using soil extract (in lieu of water) prepared by extracting one pound of garden soil in one liter of distilled water, filtering several times through S&S No. 588 paper, and bringing to volume. Cultures grown in plain nutrient broth or in the various enrichment broth media have been found to produce uniformly more luxuriant and resistant crops of spores when the garden soil extract is employed.

Loops of size 3 surgical silk suture are employed as carriers. The standard loops are prepared by wrapping the silk around an ordinary pencil three times. The coil so formed is then slipped off the end of the pencil and held firmly with the thumb and index finger. Another piece of suture is passed through the coil, knotting it, and tying it securely. The ends of the coil and the knotting suture are then sheared off to within 1/16 of an inch. This provides an over-all length of approximately $2\frac{1}{2}$ inches of suture in a two loop coil that can be conveniently handled in ordinary aseptic transfer procedures.

After exposure to the control chemical, the volume and reaction of any subculture media employed with the carriers must be carefully adjusted or the media buffered so that the hydrochloric acid carried over does not reduce the *p*H to a level too low to permit the growth of the spores which survive. Fluid thioglycollate medium can be used successfully with most *Clostridia* and *Bacilli* if 20 ml of *N* NaOH is added to each liter of medium prior to dispensing for sterilization.

For routine evaluations, strains of the non-pathogens *Cl. sporogenes* and *B. subtilis* are frequently employed, but other pathogenic and non-pathogenic species may be used. The *Bacilli* are grown in nutrient broth made up to contain 10.0 g of Armour's peptone, 5.0 g of Difco Beef Extract, and 5.0 g of sodium chloride in each liter of soil extract. The *p*H is adjusted to 6.9 and dispensed in 10.0 ml quantities in 20×150 mm test tubes; the tubes are plugged with cotton and sterilized in the autoclave at 15 lbs. for 20 min. The *Clostridia* are grown in an enrichment broth made up by adding 1.5 g of Difco meat-egg medium and 10.0 ml of soil extract to individual 20×150 mm. test tubes. The *p*H of the soil extract should be at least 5.2. All tubes are then plugged with cotton and sterilized in the autoclave at 15 lbs. pressure for 20 min. All cultures are incubated at 37°C. for 72 hours. The suture loop carriers described are placed in Petri dishes matted with filter paper and sterilized at 15 lbs. pressure for 20 min. New loops are used for each test, for the sterilization and re-use of old loops has been found to introduce variables whose nature is not understood.

Five sterile loops are placed in each 72-hour culture; the tube is agitated vigorously and allowed to stand for 15 min. The loops are then withdrawn and placed in sterile Petri dishes that have been matted with 2 sheets of filter paper and allowed to dry for 22–26 hours at room temp. All suture loop transfers are made with a 2–3″ No. 18 B&S gage nichrome wire needle having a right angle bend at the end. This is flamed in the conventional manner between transfers.

Ten ml of constant boiling hydrochloric acid is transferred into a sterile, cotton-plugged 25×150 mm lipped test tube, the tube is placed in a constant temp. water-bath at 20°C., and the acid allowed to come to temp. Four dried contaminated loop suture carriers are then transferred into the medicant tube. Theoretically this

transfer should be made simultaneously, but practically a few seconds will elapse in making 4 successive transfers. The remaining dried contaminated suture loop is then transferred to a tube of the sterile cotton-plugged subculture thioglycollate broth medium as a viability control. After 5, 10, 20, and 30 minutes, individual suture loops are withdrawn and placed in tubes of the thioglycollate subculture medium and each tube rotated vigorously for 20 seconds. All subculture tubes are incubated for one week at 37°C. Reliable readings can usually be made after 48 hours' incubation but this may not always be true.

With most cultures it is essential that the spores be dried on the carriers for a reasonable period of time. On drained and undried sutures most spores will not resist constant boiling hydrochloric acid for measurable periods. The drying period necessary depends largely on the species and strain used but a 22-26 hour period has been found adequate for most strains. The effect of drying is clearly illustrated in Table 1, which compares the results obtained with spores of both *Cl. sporogenes* and *B. subtilis* on sutures drained and dried for 5 minutes as opposed to those with contaminated sutures dried for increasing periods of time.

TABLE 1.—*Critical killing time as related to drying time*

CULTURE	AGE IN HOURS	DRYING TIME IN MINUTES	UNEXPOSED CONTROL	EXPOSURE PERIODS TO HCl IN MINUTES			
				1	2	5	10
Cl. sporogenes	72	5	+	−	−	−	−
	72	30	+	+	+	+	+
	72	60	+	+	+	+	+
	72	180	+	+	+	+	+
	72	1320	+	+	+	+	+
B. subtilis	72	5	+	−	−	−	−
	72	30	+	−	−	−	−
	72	60	+	−	−	−	−
	72	180	+	+	+	+	−
	72	1320	+	+	+	+	+

(+) = growth. (−) = no growth.

Table 1 shows that both the spores of *Cl. sporogenes* and *B. subtilis* were killed within one minute by the hydrochloric acid when the draining and drying time was only 5 minutes. By increasing the drying time to 30 minutes, resistance in the spores of *Cl. sporogenes* was increased to 10 minutes. On the other hand it was necessary to increase the drying time with *B. subtilis* to 180 minutes to obtain spores which resisted the acid for 5 minutes; drying for 1320 minutes resulted in the production of spores which resisted the acid for 10 minutes.

Lack of resistance in the undried spores of these two organisms could not be linked up with culture age. After a drying period of 5 minutes,

spores produced in 48, 72, 96, and 120 hour cultures of both species failed to survive exposure to the acid for one minute.

A special study was made with these two organisms to determine the effect of prolonged drying on the resistance of spores carried by the standardized silk suture loops. The results are shown in Table 2.

TABLE 2.—*Spore survival time in constant boiling HCl at 20°C. after prolonged drying periods*

CULTURE	AGE (HOURS)	DRYING TIME (HOURS)	UNEXPOSED CONTROLS		EXPOSURE PERIODS (MIN.)						
			No. 1	No. 2	$2\frac{1}{2}$	5	10	20	30	60	120
Cl. sporogenes	72	22–26	+	+	+	+	+	+	+	+	–
B. subtilis	72	22–26	+	+	+	+	+	+	+	–	–
Cl. sporogenes	72	166–170	+	+	+	+	+	+	+	+	–
B. subtilis	72	166–170	+	+	+	+	+	+	+	–	–

(+) =growth. (–) =no growth.

Attention is called to the fact that the spores of *Cl. sporogenes* resisted constant boiling hydrochloric acid for 60 minutes after drying for 22–26 hours and 166–170 hours. The spores of *B. subtilis* also resisted constant boiling hydrochloric acid for 30 minutes after drying for the same periods. The retention of resistance in the spores dried on the carriers for at least 7 days makes it possible to contaminate a supply of loop carriers and test a representative sample after 22–26 hours for resistance to the standard acid solution, and to hold the major number for use in examining the unknown germicides to be tested. This procedure will provide the technician with a constant supply of spores of predetermined resistance for such tests as he may have occasion to make.

In the testing of unknown germicides for sporocidal activity the volume of medicant used is the same as the volume of the standard acid control. The number of standard dried contaminated loops used is also the same. In subculturing, the media employed should contain suitable reversing substances to overcome the bacteriostatic effects of the small quantities of medicant carried over. In the absence of known suitable reversing agents for the medicant, the cultured loops should be transferred after 7 days to fresh tubes of sterile subculture media and incubated for an additional period of one week.

DISCUSSION

Curran (3) has recently pointed out that the capacity of bacterial spores to withstand destructive agents is not equaled by any other living thing. The results reported here provide an example of the magnitude of the resistance to a destructive chemical of the spores of both *Clostridia*

and *Bacilli*. The species and strains used were typical for the genera, and were not selected on the basis of prior knowledge of resistance. The absence of a measurable resistance in the undried spores to constant boiling hydrochloric acid and the marked increase in resistance as the drying time was increased to 22–26 hours, may indicate that resistance of bacterial spores to this chemical depends upon the lowering of their free or unbound water content.

SUMMARY

1. A general procedure for determining the sporocidal activity of chemical germicides by the use of spores of a predetermined resistance to a standard chemical, constant boiling hydrochloric acid, has been described.

2. The spores of *Cl. sporogenes* and *B. subtilis* have been shown to give satisfactory results in the procedure and are recommended for routine evaluations. The details of the procedure have been developed, however, so that it may be applied with other pathogenic and non-pathogenic species of both genera.

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ANALYSIS OF MANGANESE ETHYLENEBISDITHIOCARBAMATE COMPOSITIONS AND RESIDUES*

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The introduction of Manzate† fungicide has required the development of accurate methods for determining macro- and microquantities of its active ingredient, manganese ethylenebisdithiocarbamate. Assay and residue procedures based on carbon disulfide evolution have already been described in the literature for determining dithiocarbamates (1, 2, 3).

The general applicability of carbon disulfide evolution and other assay methods to dithiocarbamate fungicides has been investigated rather extensively in our laboratory over a number of years. It has been found that, although conventional carbon disulfide evolution methods are quite satisfactory for some dithiocarbamates, they are not, without modification of technique, generally applicable to all such compounds, particularly manganese ethylenebisdithiocarbamate. A modified carbon disulfide

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† Trade mark of E. I. du Pont de Nemours and Co., Inc., for fungicide based on manganese ethylenebisdithiocarbamate.

evolution procedure is the most satisfactory and most specific method for determining the active ingredient content of Manzate fungicide. A brief description of the procedure along with representative data are presented in this paper. It should not be assumed without proper evaluation that the revised technique is applicable to all dithiocarbamates.

Clark, *et al.*, (2), pointed out that ethylenebisdithiocarbamic acid is subject to two types of decompositions:

```
          H   S
          |   ||
          N———C———SH
         /
    CH2                    A    H2N—CH2—CH2—NH2 + 2CS2
    |                     |→
    |                   ——|           H
    CH2   H   S           |           N
       \  |   ||          | B       /   \
          N———C———SH      |→    CH2   H   C=S + H2S + CS2
                                 |    |  /
                                 CH2——N
```

In the published methods for assaying sodium and zinc salts of ethylenebisdithiocarbamic acid, quantitative decomposition according to reaction *A* is obtained by adding hot, dilute acid to a weighed portion of the sample, heating the mixture to a boil, and maintaining it at reflux for 15 to 20 minutes while the carbon disulfide evolved is absorbed in an alcoholic-potassium hydroxide solution. However, when hot sulfuric acid is added directly to Manzate fungicide, the yellow powder does not wet readily and floats on the surface of the digestion mixture. As the solution is brought to a boil, decomposition takes place with the evolution of large excesses of hydrogen sulfide which are reflected quantitatively by the decreased amounts of carbon disulfide obtained in the alcoholic-potassium hydroxide trap. In order to ensure quantitative evolution of carbon disulfide from manganese ethylenebisdithiocarbamate, it has been found necessary to provide adequate wetting of the Manzate in the boiling acid, and to extend the recommended digestion period from 15 to 90 minutes.

The following brief summary includes the pertinent steps of the modified technique:

METHOD

A 0.4- to 0.6-g portion of Manzate is introduced into the dry reaction flask of the digestion apparatus which has been previously assembled as shown in Figure 1. Trap 1 contains 15 ml of a 10% cadmium chloride or lead acetate soln for removal of interfering gases, and trap 2 contains 25 ml of 2 *N* methanolic-potassium hydroxide soln. Vacuum is applied, the flow rate is adjusted to ca 60 ml/min., and 50 ml of an equal vol. mixt. of water and ethyl alcohol which has previously been heated to its boiling point is introduced through the dropping funnel. After this addn, a few

bubbles of air are allowed to pass through the system, and the tip of the delivery tube is rotated in order to disperse the Manzate fungicide thoroughly. A 50 ml portion of boiling 30% sulfuric acid soln is then added, the mixt. is heated to a boil immediately, and the digestion is continued at reflux temp. for 90 min. After the reflux period, the contents of the alcoholic potassium hydroxide trap are washed into a 500 ml flask with small portions of distilled water totaling ca 200 ml. The excess caustic is neutralized carefully with acetic acid, and the xanthate formed by the reaction of carbon disulfide with methanol and potassium hydroxide is titrated rapidly with standard iodine soln to the conventional starch-iodine end point. After correcting the titer for any reagent blank, the manganese ethylenebisdithiocarbamate content of the original sample is calculated from the proper stoichiometric relations.

The results in Table 1 illustrate the importance of the modifications which have been introduced into this method. In the application of conventional carbon disulfide procedures to manganese ethylenebisdithiocarbamate, the most significant error is introduced by improper dispersion, but even in the modified procedure in which proper dispersion is obtained, it was found that the carbon disulfide yields are somewhat low if only a 15 minute digestion period is employed. This observation is illustrated in Table 2 with a series of analyses obtained on a zinc ethylenebisdithiocarbamate formulation. From such data, it was concluded that 60 to

TABLE 1.—*Assay of a Manzate fungicide sample by carbon disulfide evolution*

1. *By Conventional Procedure*

TEMP. OF ACID ADDED	TIME OF DIGESTION	MnEBD FOUND
		per cent
60°C.	15 min.	70.00, 69.04
75°C.	15 min.	70.76, 69.50
Boiling	15 min.	70.69, 69.59
Boiling	30 min.	71.51, 71.00
Boiling	60 min.	71.77, 71.70
Boiling	90 min.	73.58, 72.72

2. *By Modified Procedure*

MnEBD FOUND (USING $CdCl_2$ TRAP)	MnEBD FOUND (USING $Pb(C_2H_3O_2)_2$ TRAP)
per cent	*per cent*
81.37	82.02
81.10	81.00
81.37	
81.77	
82.55	
82.58	
81.91	
81.84	

TABLE 2.—*Variation of digestion time*

TIME OF DIGESTION	APPARENT ZnEBD FOUND
min.	*per cent*
30	64.82
45	65.39
60	67.12
90	66.81

90 minutes is required to guarantee complete recoveries of carbon disulfide.

In an investigation of the digestion details as applied to Manzate fungicide, the efficiency of the carbon disulfide absorption traps was tested. The data presented in Table 3 show that packed towers containing 25 ml of 2 *N* alcoholic-potassium hydroxide solution are adequate for complete absorption of up to twice the amount of carbon disulfide evolved in the recommended procedure, and that rates of gas flow up to four times the recommended rate can be tolerated without "leakage" into a second absorption tube.

The validity of the over-all, modified carbon disulfide procedure was established by analyzing a series of manganese ethylenebisdithiocarbamate samples which contained water as the only impurity. The results presented in Table 4 illustrate that essentially 100 per cent recoveries of the active ingredient are obtained with the outlined procedure, particularly when it is considered that part of the inconsistencies may be caused by errors in the water determinations. The summation of the analytical errors of the water and manganese ethylenebisdithiocarbamate determinations represented in Table 4 is 0.78 per cent relative, expressed on a 2 sigma basis. The most pertinent points in the technique as applied to the assay of Manzate fungicide are still under investigation, and any further improvements will be presented in a later publication.

With only minor modifications the methods described in the literature (2, 3) for determining microquantities of dithiocarbamates on food crops have been applied to residues of Manzate fungicide. The materials containing manganese ethylenebisdithiocarbamate residues are placed in the reaction flask of the apparatus shown in Figure 2, slurried with water,

TABLE 3.—*Efficiency of packed tower*

SAMPLE ANALYZED	FLOW RATE	0.1 *N* I_2 TITER*	
		FIRST TRAP	SECOND TRAP
	ml/min.	*ml*	*ml*
0.4871 g Manzate fungicide	60	30.00	0.20
Pure CS_2	60	59.92	0.20
Pure CS_2	60	42.93	0.20
Pure CS_2	224	46.90	0.20

* Reagent blank =0.20 ml.

TABLE 4.—*Analysis of MnEBD samples containing water as the only impurity*

SAMPLE NO.	RESULTS OBTAINED
1	MnEBD = 90.48%
	H_2O = 9.45%
	Total = 99.9 %
2	MnEBD = 89.49%
	H_2O = 10.95%
	Total = 100.4 %
3	MnEBD = 88.43%
	H_2O = 11.7 %
	Total = 100.1 %
4	MnEBD = 88.86%
	H_2O = 10.95%
	Total = 99.8 %
5	MnEBD = 87.7 %
	H_2O = 11.9 %
	Total = 99.6 %
6	MnEBD = 87.3 %
	H_2O = 11.9 %
	Total = 99.2 %
7	MnEBD = 88.4 %
	H_2O = 11.7 %
	Total = 100.1 %
8	MnEBD = 90.4 %
	H_2O = 9.3 %
	Total = 99.7 %

heated to a boil, and treated with boiling 2 *N* sulfuric acid. The carbon disulfide evolved is absorbed in an alcoholic solution of copper acetate and diethylamine, and is determined by measuring the intensity of the yellow color of the copper diethyldithiocarbamate formed. Using this procedure, 30 to 35 per cent recoveries are obtained when known quantities of manganese ethylenebisdithiocarbamate are added to untreated crop

pulps and 75 to 80 per cent when MnEBD is added to water scrubbings from untreated crops. Using the original procedure, in which hot acid is added to the sample being analyzed and the acidic mixture is brought to a boil, the usual recoveries are in the range of only 20 per cent of theoretical.

Sufficient data have been accumulated to show that manganese ethylenebisdithiocarbamate residues behave in a manner similar to other dithiocarbamate residues. In general, manganese ethylenebisdithiocarbamate contents, ranging from 0.05 p.p.m. to 8.0 p.p.m., have been encountered

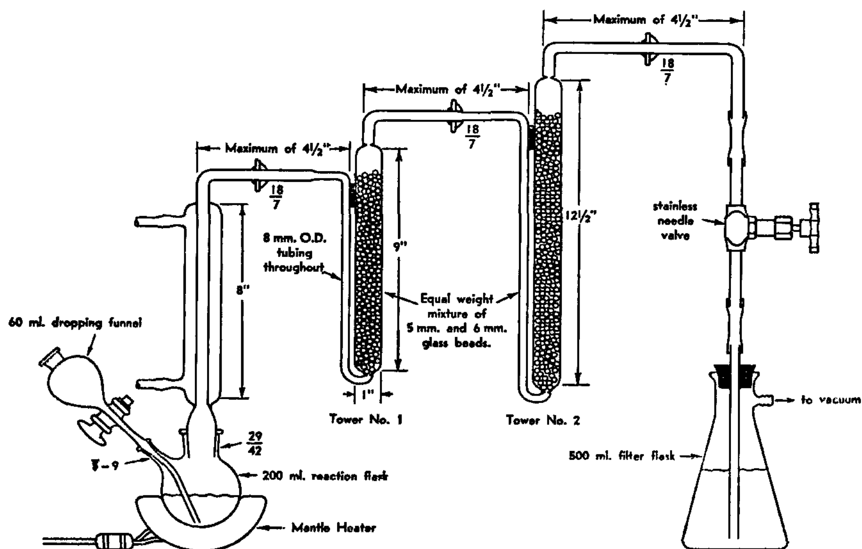

FIG. 1—Distillation apparatus for dithiocarbamate determination.

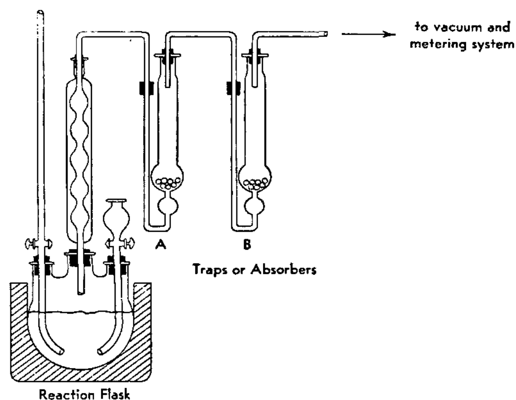

FIG. 2.—Decomposition-absorption apparatus for dithiocarbamate determination.

TABLE 5.—*MnEBD residue levels on tomatoes*

INTERVAL BETWEEN LAST APPLICATION AND SAMPLING	APPROXIMATE POUNDS MANZATE FUNGICIDE APPLIED (BY SPRAY) PER A.	NUMBER OF APPLICATIONS	RESIDUE LEVEL AS P.P.M. MnEBD
Less than 1 day	3	1	1.3
	3	1	0.5
	3	1	0.8, 0.4
	5	1	0.8
	4	1	1.1, 1.0
	2.5	17	0.4
	2.5	20	0.4
5 to 7 days	4	1	0.09
	2.25	7	0.11, 0.11, 0.04, 0.06
	2.5	16	0.3
	2.5	19	0.08

TABLE 6.—*Effect on MnEBD residue levels of time interval between last application and sampling*

CROP	APPROXIMATE POUNDS OF MANZATE FUNGICIDE APPLIED (BY SPRAY) PER A.	NUMBER OF APPLICATIONS	TIME INTERVAL (DAYS)	POST-HARVEST TREATMENT	RESIDUE LEVEL, P.P.M. MnEBD
Squash	2	3	7	None	0.07
		4	0	None	0.3
			7	None	0.08
		5	0	None	0.4
			7	None	0.09
		6	0	None	0.2
			7	None	0.06
		7	0	None	0.5
			0	Washed	0.07
Cucumber	2	4	7	None	0.00
		5	0	None	0.05
			7	None	0.00
		6	0	None	0.07
			7	None	0.02
		7	0	None	0.2
			0	Washed	0.00

on crops harvested within 1 day of the final treatment. These values are further reduced by allowing several days to elapse between the final spray and harvest. Manganese ethylenebisdithiocarbamate residues do not build up as the frequency or number of treatments is increased during a long growing season. Leafy or pubescent crop surfaces tend to retain higher residual levels of manganese ethylenebisdithiocarbamate than smooth surfaced crops having low surface-to-mass ratios, such as tomatoes or cucumbers. Washing and trimming operations commonly employed in processing fresh vegetables reduce these residues significantly, and no manganese ethylenebisdithiocarbamate has been detected in any of the canned vegetables analyzed to date.

Representative results upon which these conclusions were based are presented in Tables 5, 6, and 7. Low residues of Manzate fungicide were obtained on tomatoes (which exhibit low surface-to-mass ratios) even when a large number of treatments were employed and harvest occurred within 1 day of the final treatment (Table 5). These data also illustrate the decrease in such residues when several days are allowed to elapse between the final spray and harvest. In Table 6 the effect of time intervals (up to seven days) in reducing the quantity of residue is illustrated. The effectiveness of washing practices in reducing residue levels of treated

TABLE 7.—*Effect on MnEBD residue levels of various post-harvest treatments of the sample*

CROP	APPROXIMATE POUNDS OF MANZATE FUNGICIDE APPLIED (BY SPRAY) PER A.	NUMBER OF APPLICATIONS	TIME INTERVAL (DAYS)	POST-HARVEST TREATMENT	RESIDUE LEVEL, P.P.M. MnEBD
Peppers	4	3	4	None	0.5
			4	Washed	0.07
		4	0	None	3.2
			0	Washed	0.09
			7	None	0.32
		5	0	None	3.19
			0	Washed	0.00
			7	None	0.16
		6	0	None	5.2
			0	Washed	0.09
Celery	3	12 to 18	7	Stripped & trimmed	6.33 to 7.96
			7	Stripped, trimmed, & washed	1.75 to 2.80

squash and cucumbers is also shown. The data in Table 7 show the reduction in manganese ethylenebisdithiocarbamate residue levels obtained by washing peppers. In addition, the levels encountered on one of the most retentive crops, celery, are illustrated, along with the reductions in residues which are obtained by washing and trimming this particular crop. Again in this table, the decrease in manganese ethylenebisdithiocarbamate residues obtained by allowing a week to elapse between the final treatment and harvest is shown.

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AN ANALYTICAL SYSTEM FOR DETERMINING PHOSPHORUS COMPOUNDS IN PLANT MATERIALS*

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Increasing recognition of the importance of phosphorus compounds in plant materials, from the standpoint of both nutritional and industrial use, emphasizes the need for a reliable system of analytical methods for their determination. Procedures for the determination of total, inorganic, phosphatide, phytin, and acid-soluble phosphorus have been adapted and tested in the analysis of cottonseed. From the values obtained by these procedures, the phosphorus present as nucleic acid or phosphoproteins and as carbohydrate esters of phosphoric acid may be calculated. These derived values are subject to the accumulated errors of the determined values.

Since the final evaluations of total, phytin, and acid-soluble phosphorus involve use of a reduced molybdate colorimetric method, and inorganic and phosphatide phosphorus determinations utilized a colorimetric method involving extraction of a molybdenum blue complex with isobutyl alcohol, these two colorimetric methods are described in some detail. The specific analytical procedures used for determining phosphorus for each type of compound are then given. The sample weights and aliquot factors specified in the procedures are those which have been found to be adequate for the analysis of cottonseed kernels. Application to other plant materials

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may require adjustments in both sample weights and aliquots used. Data are presented showing the results that can be obtained by these methods.

REDUCED MOLYBDATE COLORIMETRIC METHOD

REAGENTS

(a) *Sulfuric acid.*—Concentrated, reagent grade.

(b) *Hydrogen peroxide.*—30%, reagent grade.

(c) *Sodium hydroxide.*—3.60 *N*. Dilute 195 ml of 1+1 sodium hydroxide (made from reagent grade alkali low in phosphorus and stored in a paraffin-lined bottle) to one liter with distilled water; store in a paraffin-lined bottle.

(d) *Sodium hydroxide.*—1 *N*. Dilute 278 ml of 3.60 *N* sodium hydroxide to one liter with distilled water and store in a paraffin lined bottle.

(e) *Sodium alizarin sulfonate indicator.*—0.2% aqueous solution.

(f) *Reduced molybdate.*—Concentrated reagent. Weigh 39.12 g of reagent grade molybdic anhydride (MoO_3) into a two liter, round-bottom Pyrex flask with two necks. Add 800 ml of concentrated sulfuric acid and introduce a thermometer through one neck and a glass stirrer through the other. Place the flask in an electrically heated mantle and bring to a temperature of 150°C. with stirring. Hold at this temperature until solution is complete (1.5–2.0 hrs), as indicated by the appearance of a clear, greenish-colored solution. Add quantitatively 2.20 g of powdered molybdenum metal (99.5% Mo) and hold at 150°C. with stirring until solution is again complete. This requires about 2 hours. Cool the deep blue solution, transfer quantitatively to a one liter volumetric flask using concentrated sulfuric acid to rinse the flask, dilute to volume with concentrated sulfuric acid, and mix well. Store the reagent in stoppered Pyrex bottles protected from contamination by dust. The reagent is 36 *N* in sulfuric acid, approximately 0.1 *N* as a reductant, and is stable for several years.

(g) *Reduced molybdate, dilute solution.*—Pipet 10 ml of the concentrated reagent into a 100 ml volumetric flask containing about 50 ml of distilled water. Because of the viscosity of the reagent, wash the inside of the pipet with distilled water into the flask. Cool to room temperature and dilute to volume with distilled water. Prepare the diluted reagent as needed as it is not stable for more than 6–8 hours.

(h) *Standard phosphate stock solution.*—Recrystallize A.C.S. grade monobasic potassium phosphate (KH_2PO_4) three times from water, dry at 110°C., and store in a desiccator over concentrated sulfuric acid. Dissolve 4.3929 g of the dry salt in 300 ml of water and 200 ml of *N* sulfuric acid contained in a 1 l volumetric flask, add several drops of 0.1 *N* potassium permanganate, and dilute to volume with distilled water. This stock solution, 1.0 mg phosphorus per ml, is stable.

(i) *Standard phosphate solution.*—0.01 mg phosphorus per ml. Dilute the stock solution to obtain a solution containing 0.01 mg phosphorus per ml. This solution is unstable and should be prepared as needed.

DIGESTION OF SAMPLE

Weigh sufficient sample, not to exceed 0.5 g of solid material, or pipet a suitable aliquot of extracts, to contain not more than 1.5 mg of phosphorus, into a 100 ml Kjeldahl flask. (Porcelain micro beakers are convenient for weighing solid samples.) Add 3 ml of concentrated sulfuric acid, conveniently added from an automatic burette, and two 6-mm glass beads. Heat until the organic matter chars and a homogeneous solution is obtained. Add 3–4 drops of 30% hydrogen peroxide down the neck of the flask. (Caution: Keep the mouth of the flask pointed away from the face and swirl after the addition of each drop.) Heat until colorless, repeating the

addition of peroxide if necessary. Five to ten drops of peroxide are usually required. Heat for 10 minutes after the last addition of peroxide. Cool, add about 20 ml of water and boil for 5 min. to remove any remaining peroxide and to insure conversion of the phosphorus to the ortho form. Cool, transfer quantitatively to a 100 ml volumetric flask, dilute to volume with distilled water, and mix well.

COLORIMETRIC DETERMINATION OF PHOSPHORUS

Pipet a suitable aliquot of the digested sample, containing not more than 0.12 mg of phosphorus, into a 100 ml volumetric flask with a mark at 70 ml volume. Add sufficient 3.60 *N* sodium hydroxide to approximately neutralize the acidity (see notes), two drops of indicator, and adjust with *N* acid and *N* alkali until one drop of the acid turns the solution yellow. Dilute to 70 ml with distilled water. Prepare a blank containing the same amount of 3.60 *N* alkali as used for the sample and adjust the acidity in the same manner. Pipet 10 ml of the reduced molybdate reagent into the sample and blank, mix by swirling, and place the flasks in a boiling water bath for 30 min. Remove from the bath, cool to room temperature in a cold water bath, dilute to volume with distilled water, and mix well.

Determine the transmittance of the sample using a photoelectric colorimeter equipped with a glass color filter having a transmission maximum in the range of 660–720 mμ (see note 4), using the reagent blank to set the instrument at 100% transmission. If a spectrophotometer is available, measurements should be taken at 820 mμ. Determine the mg of phosphorus in the sample aliquot by reference to the standard curve, obtained as described below, and multiply by the aliquot factor to determine mg of phosphorus in the sample.

Calibration curve.—Pipet 0, 1, 2, 3, 4, 5, 6, 7, 8, 10, and 12 ml aliquots of the dilute phosphate solution (0.01 mg P per ml) into 100 ml volumetric flasks, add two drops of indicator, one drop of *N* sulfuric acid, and dilute to 70 ml volume. Add 10 ml of reduced molybdate reagent and proceed as outlined above for the sample aliquot, using the 0.0 mg phosphorus standard to set the instrument at 100% transmission.

Plot the logarithms of the transmittance values for the standards against the known concentrations of phosphorus to obtain the calibration curve. As an alternative, the values for the logarithms of the transmittance of the standards may be treated statistically by the method of least squares to obtain the regression equation (3). Substitution of the value of log. transmittance for an unknown in the regression equation gives the mg. of phosphorus in the 100 ml volume in which the color is developed.

NOTES

1. The salt concentration (Na_2SO_4) does not affect the intensity of the developed blue color until 5 ml or more of 3.60 *N* sodium hydroxide is used to neutralize the acidity of the solutions. This allows any suitable aliquot, not exceeding 10 ml, to be used for color development. When aliquots in excess of 10 ml are required, standards containing the same amount of 3.60 *N* alkali should be used.

2. The calibration curve need be determined only once for each batch of concentrated reduced molybdate reagent, as the standards have been found to be highly reproducible and the concentrated reagent to remain unchanged. (Calibration curves for a single bath of concentrated reduced molybdate reagent were checked periodically over a period of four years and were found to be identical, indicating no change in the reagent over this period.)

3. Standard curves for different batches of reduced molybdate reagent have been found to be remarkaby constant. It is advisable, however, to prepare a standard

curve for each batch of concentrated reduced molybdate in order to eliminate any errors in the preparation of the reagent.

4. The molybdenum blue complex developed exhibits a single broad absorption band with maximum at 820 millimicrons. Since photoelectric colorimeters equipped with glass filters give inadequate response at this wave length, the measurements are made in the range of 660–720 millimicrons where the sacrifice in sensitivity is small.

5. The developed blue color is quite stable and measurements may be taken at any time within 24 hours after color development.

ISOBUTYL ALCOHOL COLORIMETRIC METHOD

REAGENTS

(a) *Molybdate reagent.*—Dissolve 50 g of ammonium molybdate in 400 ml of 10 *N* sulfuric acid and dilute to 1 liter with distilled water. Store in a paraffin-lined bottle.

(b) *Sulfuric acid, approx. 1 N.*—Dilute 114 ml concentrated sulfuric acid to 4 liters with distilled water.

(c) *Stannous chloride, stock solution.*—Dissolve 10 g of stannous chloride dihydrate in 25 ml of concentrated hydrochloric acid. Store in a small glass-stoppered brown bottle.

(d) *Stannous chloride, dilute solution.*—Dilute 1 ml of stock solution to 200 ml with *N* sulfuric acid just before use. This solution is not stable.

(e) *Isobutyl alcohol, reagent grade.*

(f) *Ethyl alcohol, 95%.*

(g) *Standard phosphate solution.*—Prepare as directed in the reduced molybdate method for phosphorus and dilute so that 1 ml contains 0.005 mg of phosphorus.

PROCEDURE

Pipet a suitable aliquot of an extract or of a solution of digested sample into a 125 ml separatory funnel with a mark at 20 ml. Add 5 ml of the molybdate reagent and dilute to 20 ml volume with distilled water. Add 10 ml of the isobutyl alcohol and shake for 2 min. Discard the aqueous layer and wash by shaking once for 0.5 minutes with 10 ml of approx. *N* sulfuric acid, again discarding the aqueous layer. Add 15 ml of dilute stannous chloride, shake for 1 min. and discard the aqueous layer. Transfer the blue isobutyl alcohol layer quantitatively to a 50 ml volumetric flask using 95% ethyl alcohol to effect the transfer. Dilute to volume with 95% ethyl alcohol.

Determine the transmittance of the blue solution against a blank containing all reagents, using a photoelectric colorimeter equipped with a filter having a transmission maximum at 720–730 mμ or a spectrophotometer at 730 mμ, at any time from 40 minutes to 19 hours after color development.

Calibration curve.—Prepare a calibration curve by pipeting suitable aliquots of the standard phosphate solution in the range of 0 to 0.045 mg of phosphorus into 125 ml separatory funnels and develop the color exactly as outlined for the determination. The logarithms of the transmittance values for the standards may be treated statistically as outlined in the reduced molybdate method. The calibration curve once determined for any instrument need not be repeated, as the standard curve has been found to be highly reproducible.

METHOD FOR PHOSPHORUS DISTRIBUTION

SPECIAL REAGENTS

(a) *Trichloroacetic acid, 0.75 N.*—Dissolve 123 g of reagent grade acid in distilled water and dilute to 1 liter. Make the reagent as needed or store in a refrigerator.

(b) *Benzene-alcohol azeotrope, B.P. 68.2°C.* (1).—Prepare by mixing 32.4 weight per cent of absolute ethyl alcohol and 67.6 weight per cent of benzene.

(c) *Ethyl alcohol, 95%.*

(d) *Ethyl alcohol, 70%.*

(e) *Magnesium nitrate solution.*—Saturated solution of magnesium nitrate hexahydrate in 95% ethyl alcohol.

(f) *Sulfuric acid, 10 N.*—Dilute 284 ml of concentrated sulfuric acid to one liter with distilled water.

(g) *Petroleum ether.*—American Oil Chemists' Society specification (2).

(h) *Hydrochloric acid, 2%, containing sodium sulfate.*—Dissolve 100 g of anhydrous sodium sulfate in about 500 ml of water contained in a 1 liter volumetric flask, add 48.5 ml of concentrated hydrochloric acid, and dilute to volume with distilled water.

(i) *Hydrocyloric acid, 0.6%, containing sodium sulfate.*—Dissolve 100 g of anhydrous sodium sulfate in about 500 ml of water contained in a 1 liter volumetric flask, add 14.5 ml of concentrated hydrochloric acid, and dilute to volume with distilled water.

(j) *Hydrochloric acid, 1 N.*—Dilute 88.5 ml of concentrated hydrochloric acid to 1 l with distilled water.

(k) *Sodium hydroxide, 5 N.*—Dilute 270 ml of 1+1 sodium hydroxide to 1 l with distilled water. Store in a paraffin-lined bottle.

(l) *Sodium hydroxide, 1 N.*—Dilute 54 ml of 1+1 sodium hydroxide to 1 l with distilled water.

(m) *Phenolphthalein indicator.*—1% solution in 95% ethyl alcohol.

(n) *Ferric chloride reagent.*—Dissolve 15.0 g of hexahydrate, $FeCl_3 \cdot 6H_2O$, in 1 *N* hydrochloric acid and dilute to 1 liter with 1 *N* hydrochloric acid.

DETERMINATIONS

Total phosphorus.—Digest 100–150 mg of the ground sample and determine the total phosphorus by the reduced molybdate method as outlined above.

$$\text{Mg total phosphorus per gram} = \frac{\text{Mg phosphorus in aliquot} \times \text{aliquot factor}}{\text{Sample weight in g.}}$$

Inorganic phosphorus.—Weigh 1 g sample into a 12.5 cm filter paper. Fold and enclose in a second paper so as to retain the sample; the second paper being left open at the top to form a thimble (2). Extract for 4 hours with petroleum ether in a Butt-type extractor. Remove the solvent by drying several hours in a steam cabinet at approximately 50°C. and grind to a fine powder in a mortar. Transfer quantitatively to a 250 ml glass-stoppered Erlenmeyer flask and add exactly 50 ml of 0.75 *N* trichloroacetic acid. Extract on a mechanical shaker for one hour and filter through ashless paper of medium retentivity, discarding the first portion of the filtrate. Pipet a 2 ml aliquot of the filtered extract into a 125 ml separatory funnel and determine the inorganic phosphorus as outlined in the isobutyl alcohol method.

$$\text{Mg inorganic phosphorus per gram} = \frac{\text{Mg inorganic phosphorus in aliquot} \times 25}{\text{Sample weight in g.}}$$

Acid-soluble phosphorus.—Use a 5 ml aliquot of the filtered trichloroacetic acid extract from the inorganic phosphorus determination and digest as outlined in the reduced molybdate method. Dilute the digested solution to 100 ml volume and use a 5 ml aliquot for colorimetric phosphorus analysis by the reduced molybdate method.

$$\text{Mg acid soluble phosphorus per g} = \frac{\text{Mg phosphorus in aliquot} \times 200}{\text{Sample weight in g}}$$

Phosphatide phosphorus.—Weigh 2 g of the ground sample into a small fritted

glass extraction thimble (25×85 mm., maximum pore size 160 mμ) containing a mat of Pyrex glass wool. Extract at a rapid rate for 2 hours in a small size Soxhlet extractor, using 60 ml of the benzene-alcohol solvent. Remove the thimble, dry in a steam cabinet at 50°C., and grind the sample to a fine powder in a mortar. Return the sample to the thimble and re-extract for 2 hours. Remove the thimble and reserve the extracted meats for use in the phytin phosphorus determination.

Remove most of the solvent from the boiling flask by means of distillation and pour the residue into a small porcelain casserole (60 ml cap.). Wash the boiling flask with two 10 ml portions of 95% ethyl alcohol, heating to boiling each time, and add the washings to the casserole. Repeat, using two 10 ml portions of 70% ethyl alcohol.

After removing most of the solvent on a steam bath, add 3 ml of magnesium nitrate solution, and heat on a hot plate at low heat until the residue is dry, then at higher heat until the sample is well charred. Ash in a muffle furnace at 400°C. until most of the carbon is burned off, raise the temperature to 600°C., and hold at this temperature until a white ash is obtained. Dissolve the cooled ash in 2 ml of 10 *N* sulfuric acid, warming if necessary, and transfer quantitatively to a 100 ml volumetric flask with the aid of distilled water. Dilute to volume with distilled water and mix well. Pipet a 2 ml aliquot of the digest into a 125 ml separatory funnel and determine the phosphorus colorimetrically as outlined in the isobutyl alcohol method.

$$\text{Mg phosphatide phosphorus per g} = \frac{\text{Mg phosphorus in aliquot} \times 50}{\text{Sample weight in g}}$$

Phytin phosphorus.—Dry the extracted sample from the phosphatide extraction in a steam cabinet and transfer quantitatively to a 250 ml glass-stoppered Erlenmeyer flask, adding the glass wool mat to the flask to insure quantitative transfer. Add exactly 100 ml of the 2% hydrochloric acid solution and extract on a mechanical shaker for 2 hours. Filter through ashless paper of medium retentivity, discarding the first portion of the filtrate.

Pipet 20 ml of the filtered extract into a 50 ml graduated conical tipped centrifuge tube. Add one drop of phenolphthalein indicator, 2 ml of 5 *N* sodium hydroxide, and adjust with 1 *N* sodium hydroxide and 1 *N* hydrochloric acid until colorless. Dilute to 25 ml volume with distilled water and add 5 ml of the ferric chloride reagent, swirling the tube during the addition. Place the tube in a boiling water bath, introduce a small stirring rod, and heat for 15 minutes with occasional stirring to promote flocculation of the ferric phytate. Cool the tube in a cold water bath for 20 minutes, wash the stirring rod with a small quantity of the 0.6% hydrochloric acid, and centrifuge the tube for 20 minutes at a minimum of 1800 r.p.m. Pour off the clear supernatant and wash the ferric phytate with 5 ml of 0.6% hydrochloric acid delivered from a pipet so as to disperse the precipitate. Wash the walls of the tube with an additional 2 ml of the 0.6% hydrochloric acid. Centrifuge the tube for 20 minutes at 1800 r.p.m. and discard the clear supernatant.

Suspend the precipitate in about 5 ml of hot distilled water, add 2 ml of 1 *N* sodium hydroxide, and heat in a boiling water bath for 15 minutes with occasional stirring. Filter the hot solution through a 7 cm ashless filter paper suitable for the filtration of gelatinous precipitates, collecting the filtrate in a 100 ml Kjeldahl flask. Wash the tube with three 5 ml portions of hot distilled water, decanting through the filter each time. Wash the paper with three additional 5 ml portions of hot water. Digest the sample as outlined in the reduced molybdate method, making the digest to 200 ml volume. Use a 5 ml aliquot for the colorimetric determination of phosphorus by means of the reduced molybdate method.

$$\text{Mg phytin phosphorus per g} = \frac{\text{Mg phosphorus in aliquot} \times 200}{\text{Sample weight in g}}$$

Nucleic acid phosphorus.—Since phytin, inorganic, and ester-type phosphorus are all included in the determination of acid soluble phosphorus, subtract the sum of this value and the value for phosphatide phosphorus from the value for total phosphorus in order to estimate the amount of nucleic acid phosphorus.

Mg nucleic acid phosphorus per g =
Mg total phosphorus − (mg acid-soluble phosphorus + mg phosphatide phosphorus)

Ester type phosphorus.—Subtract the sum of the values for phytin and inorganic phosphorus from the value for acid-soluble phosphorus in order to estimate the phosphorus which is present in the form of carbohydrate esters of phosphoric acid.

Mg ester phosphorus per g =
(Mg acid-soluble phosphorus) − (mg phytin phosphorus + mg inorganic phosphorus)

EXPERIMENTAL AND DISCUSSION

Cottonseed meats were selected as sample material for the investigation of the analytical procedures for the various types of phosphorus compounds since they offer a wide range of possible interferences. They contain oil, protein, carbohydrates, and considerable pigmentation. Appreciable quantities of total, phosphatide, and phytin phosphorus are present, as well as significant amounts of inorganic, nucleic, and carbohydrate phosphorus. The experimental results and the discussion of the analytical methods selected are outlined below with respect to each type of phosphorus determined. Nucleic acid and ester-type phosphorus are discussed in the section with acid-soluble phosphorus.

Total phosphorus.—The reduced molybdate reagent employed for the colorimetric determination of phosphorus is essentially that proposed by Gerritz (4), adapted from the work of Zinzadze (5). The procedure for the sample digestion and colorimetric analysis by the reduced molybdate method is given in detail as it differs in several respects from the method proposed by Gerritz.

Inorganic phosphorus.—The method proposed by Pons and Guthrie (6) was chosen for the determination of inorganic phosphorus in the de-fatted sample. Trichloroacetic acid, 0.75 *N*, has been shown to be the most satisfactory acid for the extraction of inorganic phosphorus from plant materials of high protein content, and the isobutyl alcohol method for colorimetric phosphorus is applicable in the presence of considerable amounts of organic phosphorus compounds (6).

The effect of the de-fatting solvent on the inorganic phosphorus content of the extracted sample was investigated, and the results are shown in Table 1. In some instances, 95 per cent ethyl alcohol extracts part of the inorganic phosphorus and leads to low values for inorganic phosphorus in the de-fatted residue. Benzene-alcohol and petroleum ether do not appear to extract any significant amounts of inorganic phosphorus.

Attempts to substitute 2 per cent hydrochloric acid for 0.75 *N* trichloro-

acetic acid for the extraction of inorganic phosphorus in petroleum ether or benzene-alcohol extracted samples were unsuccessful, as the final blue solutions obtained by the isobutyl alcohol method were turbid.

Acid-soluble phosphorus.—The extraction of plant materials and animal tissues with trichloroacetic acid, usually at low temperatures, is an accepted procedure for the separation, prior to analysis, of phosphorylated intermediates (7, 8, 9, 10). Trichloroacetic acid extracts inorganic and phytin phosphorus, as well as the glucose, fructose, and other carbohydrate esters of phosphoric acid (7, 8, 9, 10). It has been reported (11, 12, 13) that phosphorus present in the form of nucleic acid or nucleotides and as phosphoproteins is not extracted by trichloroacetic acid under conditions similar to those described.

The conditions used for the extraction of acid-soluble phosphorus, 1 hour at room temperature with 0.75 N trichloroacetic acid, do not lead to any hydrolysis of phosphorylated intermediates such as fructose diphosphate, adenylic acid, sodium β glycerophosphate, or phytin (6). Glucose-1-phosphate is hydrolyzed to some extent under these conditions. However, time extractions with 0.75 N trichloroacetic acid at room temperature and at 5°C. have indicated that such easily hydrolyzable esters are not present in cottonseed and other oilseeds (6). The presence of degradation products of the nucleic acids or nucleotides in the acid soluble fraction is not likely. Although contact with strong mineral acids for long periods of time leads to considerable alteration in the nucleic acid compounds, previous work has indicated that these alterations are not accompanied by the formation of acid-soluble phosphorus compounds (13).

The analysis for acid-soluble phosphorus, supplemented by phytin and inorganic phosphorus determinations, allows an estimation of the amount of phosphorus present as carbohydrate esters of phosphoric acid. Additionally, the phosphorus present in the form of nucleic acids and phosphoproteins can be calculated from the total, acid-soluble, and phosphatide values (13).

The effect of the de-fatting solvent on the determination of acid-soluble phosphorus in cottonseed meats is illustrated by the data shown in Table 1. The values obtained after extraction with alcohol and benzene-alcohol were lower than those for the petroleum ether defatted samples. These lower values are probably due to incomplete extraction, since it has been reported for tissues that extraction with alcohol-containing solvents inhibits the subsequent extraction of acid-soluble phosphorus (13). Consequently, petroleum ether was selected for defatting the samples prior to extraction of acid-soluble phosphorus.

Phosphatide phosphorus.—The solvents which have been proposed for the extraction of phosphatides from plant materials are either 95 per cent ethyl alcohol alone or alcohol in combination with other solvents (14).

Alcohol is required for the dissociation of the protein-phospholipid complexes, after which the phosphatides are easily soluble (14). In the case of oilseeds, 95 per cent ethyl alcohol (15, 16), alcohol-ether mixtures

TABLE 1.—*Effect of de-fatting solvent on the determination of inorganic, acid-soluble, and phosphatide phosphorus in cottonseed*

SAMPLE NUMBER	MOISTURE CONTENT	SOLVENT USED FOR DE-FATTING RAW MEATS	PHOSPHORUS FOUND, PER GRAM OF RAW MEATS				
			INORGANIC IN DE-FATTED MEATS	ACID SOLUBLE IN DE-FATTED MEATS	INORGANIC IN SOLVENT EXTRACT	PHOSPHATIDE	
						APPARENT PHOSPHATIDE IN SOLVENT EXTRACT	CORRECTED FOR INORGANIC IN SOLVENT EXTRACT
	per cent		*mg*	*mg*	*mg*	*mg*	*mg*
A	9.85	Petroleum ether	0.28	9.32	0.004	0.08	—
		Alcohol-benzene	0.27	8.82	0.017	0.64	0.62
		95% ethyl alcohol	0.26	8.85	0.060	0.67	0.61
B	9.06	Petroleum ether	0.94	10.72	0.004	0.05	—
		Alcohol-benzene	0.94	10.18	0.020	0.35	0.33
		95% ethyl alcohol	0.80	10.18	0.221	0.55	0.33
C	7.85	Petroleum ether	0.96	9.03	—	—	—
		Alcohol-benzene	0.95	8.81	—	0.52	0.52
		95% ethyl alcohol	0.74	8.59	0.270	0.78	0.51
D	7.28	Petroleum ether	0.44	11.54	None	0.04	—
		Alcohol-benzene	0.45	10.74	0.005	0.66	0.66
		95% ethyl alcohol	0.44	11.04	0.045	0.75	0.70

(17, 18), and alcohol-benzene mixtures (19, 20, 21) have been suggested as solvents for phosphatides. Ethyl alcohol, 95 per cent, has been shown to extract more phosphorus from soybeans than other solvents or combinations of solvents, although the amount of inorganic phosphorus present in the alcohol extract was undetermined (16).

In order to select the appropriate solvent for the phosphatide extraction, several samples of cottonseed meats were extracted in Soxhlet extractors with petroleum ether, 95 per cent ethyl alcohol, and an azeotropic mixture of alcohol and benzene. Adsorption of phosphatides on paper thimbles was avoided by using fritted glass thimbles for the extractions. The solvent extracts were analyzed for phosphorus content as outlined in the phosphatide procedure. In addition, parallel extractions were conducted in which aliquots of the solvent extracts were analyzed for inorganic phosphorus by the isobutyl alcohol method. The results of these experiments are presented in Table 1. Petroleum ether extracts very little phosphatide phosphorus, while 95 per cent ethyl alcohol extracts somewhat more apparent phosphatide phosphorus than does alcohol-benzene. However, a considerable portion of the inorganic phosphorus is extracted by 95 per cent ethyl alcohol, the amount varying with the level of inor-

ganic phosphorus and the moisture content of the meats. When the apparent phosphatide phosphorus is corrected for the inorganic phosphorus present in the extracts, the phosphatide phosphorus extracted by 95 per cent ethyl alcohol and by alcohol-benzene are equivalent. The alcohol-benzene azeotrope was selected for the phosphatide extraction since the amounts of inorganic phosphorus in these extracts introduces no significant error in the determination. This azeotropic mixture is similar to the 20:80 alcohol-benzene mixture proposed by Rewald (21) and Schramme (19). Four-hour Soxhlet extraction under the conditions described in the

TABLE 2.—*Recovery of phytin phosphorus*

SOLUTIONS USED	PHYTIN PHOSPHORUS			
	IN COTTON-SEED EXTRACT	AS ADDED PHYTIN-P	TOTAL PRESENT	FOUND
	mg	*mg*	*mg*	*mg*
(A) Na phytate from pure Ba-phytate	—	0.64	0.64	0.63
(A) Na phytate from pure Ba-phytate	—	1.61	1.61	1.60
(A) Na phytate from pure Ba-phytate	—	3.21	3.21	3.22
(A) Na phytate from pure Ba-phytate	—	4.82	4.82	4.93
(B) Na phytate from cottonseed extracts	—	0.96	0.96	0.93
(B) Na phytate from cottonseed extracts	—	1.91	1.91	1.91
(B) Na phytate from cottonseed extracts	—	3.82	3.82	3.86
Control—2% HCL extract of cottonseed	1.74	—	1.74	—
Control+pure phytin (A)	1.74	0.64	2.38	2.42
Control+pure phytin (B)	1.74	0.96	2.70	2.73
Control+pure phytin (A)	1.74	0.96	2.70	2.70
Control+pure phytin (A)	1.74	1.61	3.35	3.43
Control+pure phytin (A)	1.74	3.21	4.95	5.11

procedure was found to be sufficient for complete extraction of the phosphatides from cottonseed meats.

Phytin phosphorus.—The phytin phosphorus procedure is based on the methods proposed by McCance and Widdowson (22) and Young (23). The use of hydrochloric acid containing sodium sulfate was suggested by Earley (24).

In the development of the procedure, inconsistent results were obtained when attempts were made to wash the ferric phytate precipitate twice with 0.6 per cent hydrochloric acid, centrifuging between each washing. The colloidal nature of the precipitate led to cloudy centrifugates, in many instances, which produce low results. When the precipitate was washed once, as previously described, consistent results were obtained. The minimum number of washings required for removal of other phos-

phorus compounds was established by precipitating ferric phytate from several aliquots of a 2 per cent hydrochloric acid extract in the usual manner, and washing the precipitates 0, 1, and 2 times with 0.6 per cent hydrochloric acid prior to decomposition with sodium hydroxide. The solutions were analyzed for total phosphorus and the results indicated that one washing of the precipitate was adequate for removal of other phosphorus compounds which may be adsorbed on the precipitate.

Attempts were made to utilize trichloroacetic acid extracts for the phytin phosphorus determination as suggested by Sarma (25). Severe foaming during the heating period, and cloudy centrifugates led to low results in many instances. When obviously low results were disregarded, the phytin phosphorus values were in good agreement with those obtained by use of

TABLE 3.—*Phosphorus distribution in selected samples*

SAMPLE	PHOSPHORUS DISTRIBUTION PER GRAM OF MOISTURE-FREE MATERIAL						
	TOTAL	ACID SOLUBLE	INORGANIC	PHOSPHATIDE	PHYTIN	NUCLEIC	ESTER TYPE
	mg	*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
Cottonseed kernels 1	13.15	11.69	0.41	0.73	10.69	0.73	0.59
Cottonseed kernels 2	11.73	10.49	0.24	0.71	9.04	0.53	1.21
Cottonseed kernels 3	10.75	9.55	0.96	0.57	8.44	0.63	0.15
Cottonseed kernels 4	8.97	7.71	0.16	0.67	6.60	0.59	0.95
Cottonseed kernels 5	8.26	6.98	0.16	0.70	6.20	0.58	0.57
Alfalfa meal	2.83	1.92	1.26	0.47	0.06	0.44	0.60
Rice bran	25.48	22.50	0.62	0.28	21.36	2.70	0.52
Sesame seed	6.37	5.79	0.22	0.30	5.47	0.28	0.10
Wheat	3.50	3.05	0.29	0.07	2.66	0.38	0.10
Corn	2.81	2.59	0.32	0.04	2.09	0.18	0.18
Peanut kernels	3.34	2.95	0.24	0.30	2.34	0.09	0.37

2 per cent hydrochloric acid. Due to these operational difficulties with trichloroacetic acid extracts, 2 per cent hydrochloric acid was selected as the extractant for phytin phosphorus.

Comparable values for phytin phosphorus were obtained when petroleum ether, 95 per cent ethyl alcohol, or alcohol-benzene was used for defatting the meats prior to analysis for phytin phosphorus.

The recovery of phytin phosphorus was determined both in the presence and absence of cottonseed extracts. Two standard solutions of sodium phytate were used for the recovery experiments. One was prepared from pure barium phytate obtained from commercial phytin as directed by Anderson (26). Another solution was prepared from several 20 ml aliquots of cottonseed extracts by precipitation of ferric phytate, washing, and decomposition with sodium hydroxide in the usual manner. After filtration, the sodium phytate solutions from the cottonseed extracts were combined.

The phytin phosphorus content of each of the two solutions of sodium phytate was considered to be the difference between the total and inorganic phosphorus contents. Aliquots of these solutions were analyzed by the phytin phosphorus procedure both in the presence and absence of cottonseed extracts. The recovery data shown in Table 2 indicate satisfactory recovery of added phytin phosphorus. The use of sodium phytate prepared from cottonseed extracts and the satisfactory recovery of phytin phosphorus from this solution indicate that the ferric phytate precipitate is not contaminated with any significant amounts of inorganic phosphorus or other organic phosphorus compounds.

The application of the procedures for the determination of the types of phosphorus compounds in cottonseed is illustrated by the data in Table 3. All values are calculated on a moisture-free basis and are expressed as mg of phosphorus per gram of meats. The samples listed were selected to illustrate the differences in the amounts of the various types of phosphorus compounds which may be present in plant materials.

SUMMARY

Analytical procedures for the determination of total, inorganic, acid-soluble, phosphatide, and phytin phosphorus are described. Two colorimetric phosphorus methods used in the final evaluation of these different types of phosphorus compounds are given in some detail. Phosphorus present as nucleic acids or nucleotides and as carbohydrate esters may be calculated from the analytical data for the other types of phosphorus.

Experimental evidence substantiating the choice of these phosphorus methods and their validity when applied to cottonseed is presented.

Although tested for validity and precision in the analysis of cottonseed, these procedures should be equally suitable for the analysis of other oilseeds and plant materials.

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THE CHEMICAL COMPOSITION OF CERTAIN GRADES OF TYPE 11, AMERICAN FLUE-CURED TOBACCO

RELATIONSHIP OF COMPOSITION TO GRADE CHARACTERISTICS*

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INTRODUCTION

In the comprehensive system for the classification and grading of American leaf tobacco developed by the United States Department of Agriculture (42), flue-cured tobacco is divided into four types, namely, 11, 12, 13, and 14. The tobacco of each of these four types is divided into groups designated by the letters A (Wrappers), B (Leaf), C (Cutters), H (Smoking Leaf), X (Lugs), P (Primings), and N (Nondescript), and each of the groups is divided into individual qualities which are indicated by Arabic numerals, followed by one letter which indicates color. In brief, for flue-cured tobacco, the Federal grade assigned depends normally upon three factors: (1) group, (2) numerical quality designation, and (3) color. In some cases a fourth or a special factor is added to designate some unusual characteristic. Numerical quality is based on such attributes of the leaf as thickness, length, width, texture, and wholeness or freedom from physical injury. Thus, C2L designates a leaf having the characteristics of those

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leaves normally produced along the median region of the stalk, of second quality and having a lemon-yellow or the lightest color of the type, and X4F is the grade designation of tobacco having the general appearance and other physical properties of the leaves usually produced on the lower portion of the stalk, of fourth quality, and of an orange or the medium color of the type.

Old Belt, or Type 11, tobacco is one of the four types of flue-cured tobacco produced in the United States. It is used largely for the manufacture of cigarettes, and is produced in the Piedmont region of Virginia and North Carolina. The purpose of the present paper is to direct attention to the differences in chemical composition of certain grades of Type 11 flue-cured tobacco, and to point out possible relationships between chemical composition and the several properties and quality characteristics of the leaf within each grade.

REVIEW OF LITERATURE

There is at present an extensive literature on the chemical composition of cigarette tobacco, but this is devoted largely to Russian and Turkish types and includes the Greek, Romanian, and Bulgarian (5, 19, 39, 41). Although from the standpoints of quantity produced and dollar value, flue-cured tobacco is our most important tobacco crop, our knowledge of the chemical composition of the various types of this class of tobacco is rather limited. Among the early investigators of the chemical composition of American flue-cured tobacco may be mentioned Moore (24) and Carpenter (6). Moore reported on the chemical composition (as determined by conventional methods) of "bright wrapper" tobacco produced in Granville County, N. C. (presumably corresponding to what is now designated as Type 11 flue-cured tobacco). Carpenter analyzed yellow tobacco from Granville County, N. C., which had been cured by two different methods and sorted into scrap, trash lug, best lug, sand lug, best lug (cutters), first and second grade wrappers, bright tips, and black tips. No attempt was made to correlate the chemical findings of the various grades of tobacco under investigation.

Garner, Bacon, and Bowling (14) reported on the chemical composition of two domestic cigarette tobaccos, namely, flue-cured (grown in Granville County, N. C.,—presumably Type 11) and Maryland (Type 32), as well as two cigar types of tobacco, Pennsylvania cigar filler (Type 41, and Connecticut Broadleaf (Type 51). Inasmuch as these investigators were interested primarily in pointing out the differences in chemical composition existing between cigarette and cigar tobaccos as such, as well as between the types of these two classes of tobacco, no attempt was made to determine the characteristic differences between the various grades of the types of tobaccos examined.

Darkis, Dixon, and Gross (9) determined some of the groups of organic

constituents of Types 11, 12, 13, and 14 flue-cured tobaccos. In the case of Type 11, the tobacco was subdivided into the Durham, Winston, and Danville sub-types, and these were examined separately. In all cases, analyses were made on a medium grade of redried unstemmed tobacco used for the manufacture of cigarettes. The several types of tobacco examined were not classified into U. S. grades. The principal difference in chemical composition was found between the Coastal Plain tobaccos (Types 12, 13, and 14) on the one hand, and the Piedmont tobaccos (Type 11) on the other. Thus, the percentages of total nitrogen was somewhat higher, and the nicotine, total nonvolatile acidity, and petroleum ether extracts were considerably higher in the Piedmont tobaccos, whereas the percentages of the sugars were higher in the Coastal Plain tobaccos. The chief difference in chemical composition between the three types of Coastal Plain tobaccos was in the percentages of sugars and total nitrogen. Type 14 was found to have the highest percentage of sugars; next in order was Type 13, and Type 12 had the lowest sugar content. In the case of the percentage of total nitrogen this relationship was reversed.

Darkis, Dixon, Wolf, and Gross (10) studied the composition of Durham flue-cured (Type 11) tobacco produced in four different crop years under varying weather conditions and determined the relationship between chemical composition and stalk position of the leaf. The tobaccos produced in three crop years were sorted by a farm grader, and the tobacco from the fourth crop year was analyzed as primings or pullings from different levels of the stalk. In all cases, the entire leaf was analyzed. The percentage of nicotine was low and the potassium high in the lower leaves. whereas in the upper leaves the reverse was true. The percentage of total sugars was highest in the middle leaves, and appeared to be inversely related to the total acids. The total nitrogen, water-soluble nitrogen, and α-amino nitrogen were all relatively high in the lower and upper leaves and all these nitrogen fractions showed an inverse relationship to the content of total sugars. The percentage of soluble ash was high in the lower leaves, decreased to a minimum in the middle leaves, and increased again in the upper leaves.

According to Ward (44), who was working with Canadian flue-cured tobacco, the quality of tobacco is directly related to the percentage of sugars in the leaf. In the case of New Zealand flue-cured tobacco, Blick (4) found that there was a fairly good agreement between quality and the ratio of total sugars to total nitrogen.

A search throughout the literature has disclosed the fact that no attempt had previously been made to determine the chemical composition of the various standard grades of flue-cured tobacco (classified according to the system developed by the U. S. Department of Agriculture), and to correlate the chemical composition of the various grades with the several characteristics or quality factors which determine the grade and general usefulness of tobacco.

MATERIALS

Selection and Preparation of Samples.—Twelve samples of the 1948 crop of Type 11 Old Belt flue-cured tobacco were chosen to represent characteristic differences in the groups and certain qualities and colors within each group. It was planned to select uniform lots of farm-sorted tobaccos, of each group, which would be two grades apart in quality as well as color, or which would have been represented by the third and fifth qualities and by the L and R colors. It was also planned to select the samples of each grade from 10 different farm lots and blend them together to minimize the effect of differences in soil, climate, and cultural practices. The somewhat limited number of uniform lots representative of these selected grades, available on the auction market at the time, prevented the carrying out of this plan in its entirety. Samples of uniformly sorted farm lots were selected by competent judges of this type of tobacco from different warehouses on the Danville, Va., auction market to represent 12 grades as follows: B3L, 10 lots; B3R, 8 lots; B5L, 7 lots; B5GR, 9 lots; H3L, 10 lots; H5L, 6 lots; H5R, 9 lots; C3L, 8 lots; C5L, 10 lots; X3L, 9 lots; X5L, 9 lots; and P5L, 3 lots. Substantially equal portions of the several farm lots of each grade were commingled to form a representative sample of the type and grade. The stems or midribs were removed by hand, and only the strip or web portions of the leaves were used for analysis. Each sample was then dried at room temperature, ground in a Wiley mill equipped with a 1 mm. sieve, thoroughly mixed, and stored in a 2-quart air-tight Mason jar.

METHODS

All analyses were made in duplicate on the dried (at room temperatures) and ground tobacco and the results (except for sand) were calculated on the basis of moisture-free and sand-free material. The percentage of sand was calculated on the moisture-free basis.

Moisture.—A weighed (1 to 2 g) sample of the tobacco, which had been dried at room temperature, was placed in an aluminum moisture dish and dried for 4 hours at 100°C., and the loss in weight was calculated as percentage of moisture.

Sand.—The percentage of sand was determined by the A.O.A.C. Method (1, p. 94).

Ash (sand-free).—The percentage of total ash was determined by heating a weighed sample (2 g) for 2 hours at 600°C. in an electric muffle furnace, provided with a temperature controller, and weighing the inorganic residue. From the percentage total ash thus determined, the percentage of sand was deducted and the result recorded in Table 1 as "Ash (sand-free)."

Petroleum Ether Extractives.—The sample (equivalent to 5 g of moisture-free tobacco), contained in a fritted-glass extraction thimble, was extracted for 8 hours with petroleum ether (boiling range 30–65°C.) in a Soxhlet extraction apparatus. The residual material was first dried on the steam bath until the odor of petroleum ether could no longer be detected, and it was then dried for 4 hours at 100°C. and from the loss in weight, the percentage of petroleum ether extractives was calculated.

Ether Extractives.—The residual tobacco from the petroleum ether extraction was extracted with ether for 8 hours in a Soxhlet extraction apparatus, the loss in

TABLE 1.—*Composition of several grades of flue-cured tobacco, Type 11 (1948 crop)**

U. S. GRADE	SAND	ASH (SAND-FREE)	PETROLEUM ETHER EXTRACTIVES	ETHER EXTRACTIVES	ALCOHOL EXTRACTIVES	TOTAL NITROGEN	PROTEIN	NICOTINE	TOTAL REDUCING SUBSTANCES (AS GLUCOSE)	REDUCING SUGARS (AS GLUCOSE)
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
B3L	1.16	9.77	4.34	1.85	45.77	1.95	5.91	2.36	24.8	21.5
B5L	1.06	9.30	4.04	1.91	45.23	1.93	5.84	2.06	22.0	18.5
H3L	1.42	10.22	5.36	2.46	45.44	1.95	5.64	2.36	26.1	22.9
H5L	1.45	9.68	5.53	2.00	42.75	1.97	6.09	1.99	24.5	20.7
C3L	2.90	10.97	7.53	1.76	42.22	1.92	5.53	2.27	23.0	20.4
C5L	6.06	13.54	6.63	3.14	35.04	2.16	6.25	2.22	15.3	14.5
X3L	8.72	15.24	5.48	2.34	33.93	2.19	6.26	2.10	13.9	11.1
X5L	10.08	17.55	6.12	1.98	26.09	2.51	6.98	2.14	6.3	5.1
P5L	10.62	20.88	5.05	1.66	21.65	2.62	7.27	1.52	3.6	2.2
B3R	0.54	11.29	4.81	2.07	40.05	3.03	6.79	4.75	13.4	11.3
B5GR	0.48	10.05	4.39	2.29	37.60	3.14	7.97	3.58	12.6	10.9
H5R	0.62	10.85	6.97	1.83	30.68	2.54	7.00	3.02	9.2	7.1

U. S. GRADE	SUCROSE	TOTAL SUGARS	DEXTRIN	STARCH	TOTAL PECTIC SUBSTANCES (AS CALCIUM PECTATE)	PENTOSANS	CELLULOSE	LIGNIN	METHOXYL IN ASH-FREE LIGNIN
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
B3L	1.9	23.4	0.8	2.7	9.0	2.36	8.0	2.05	2.72
B5L	1.9	20.4	0.5	2.6	9.7	2.67	9.2	2.63	2.20
H3L	1.0	23.9	0.8	3.3	8.3	2.54	8.2	2.30	—
H5L	0.8	21.5	0.6	4.2	8.7	2.61	8.9	2.60	2.67
C3L	0.7	21.1	0.6	4.3	8.8	2.31	8.3	2.65	2.69
C5L	0.2	14.7	0.5	1.6	9.2	2.75	10.3	3.62	2.13
X3L	1.1	12.2	0.6	0.7	8.5	3.29	10.8	3.34	2.34
X5L	0.2	5.3	0.6	0	8.7	3.11	11.1	4.37	2.47
P5L	0.4	2.6	0.6	0	9.2	3.24	13.6	4.16	3.27
B3R	0.5	11.8	0.9	1.2	9.7	2.74	10.3	2.63	2.77
B5GR	0.6	11.5	0.8	1.4	8.8	2.85	9.3	3.10	2.41
H5R	0	7.1	0.9	1.9	9.3	3.34	11.5	4.76	2.29

U. S. GRADE	ESTER METHOXYL IN TOBACCO	ETHER METHOXYL IN TOBACCO	METHOXYL IN TOBACCO (TOTAL)	POLYPHENOLS (AS GLUCOSE)	TANNINS	OXALIC ACID	CITRIC ACID	*l*-MALIC ACID	RESINS AND WAXES	*p*H
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
B3L	0.82	0.13	0.95	2.2	2.6	1.44	0.53	1.8	8.85	5.1
B5L	0.86	0.14	1.00	1.9	2.5	1.76	0.66	1.4	9.34	4.9
H3L	0.84	0.13	0.97	1.2	1.9	1.56	0.48	2.8	9.59	5.2
H5L	0.85	0.13	0.98	1.9	2.1	1.50	0.53	2.5	8.92	5.1
C3L	0.78	0.15	0.93	1.4	2.0	1.48	0.49	2.6	9.62	5.05
C5L	0.83	0.18	1.01	0.3	2.1	1.53	0.86	3.5	10.27	5.2
X3L	0.82	0.18	1.00	1.1	1.5	1.93	1.36	4.8	10.47	5.1
X5L	0.80	0.20	1.00	1.1	1.5	2.81	2.66	6.2	10.05	5.25
P5L	0.78	0.20	0.98	0.8	1.0	2.95	3.90	7.0	10.07	5.5
B3R	0.93	0.12	1.05	1.1	2.0	1.73	1.11	3.6	10.23	5.0
B5GR	0.99	0.14	1.13	1.2	2.0	1.82	0.78	2.0	9.79	4.8
H5R	1.00	0.21	1.21	1.0	2.5	2.39	0.75	1.3	11.07	4.9

* Analytical data, except percentage sand, were calculated on a moisture-free and sand-free basis. The percentage sand was calculated on a moisture-free basis.

weight was determined and calculated on the basis of the original unextracted tobacco.

Alcohol Extractives.—The residue from the ether extraction was similarly extracted for 8 hours with 95 per cent ethanol, and the percentage loss in weight was calculated on the basis of the original unextracted tobacco.

Total Nitrogen.—The percentage of total nitrogen was determined by the official Kjeldahl-Wilforth-Gunning method, modified to include nitrate nitrogen (1, p. 13). The final digestion with mercuric oxide, potassium sulfate, and sulfuric acid was continued for 4 hours.

Protein.—The method of determining percentage of protein was essentially that of Mohr (23). The analysis was carried out as follows: the sample (equivalent to 2 g of moisture-free material) was boiled for 10 minutes with 75 ml of 0.5% acetic acid solution, the mixture was filtered, and the residue was washed with a hot 0.5% acetic acid solution until the filtrate was colorless (about 450 ml). The nitrogen in the residue was determined by the Kjeldahl-Wilforth-Gunning method (1, p. 13) with mercuric oxide as catalyst, and the percentage of nitrogen found was multiplied by the conventional factor 6.25 to give the percentage of protein.

Nicotine.—Nicotine was determined by the official A.O.A.C. method (1, p. 69). In some cases, determinations were also made by the method of Chamberlain and Clark (8). The results obtained by these two methods did not vary materially.

Total Reducing Substances.—A modification of the Pyriki (31) method was used for the extraction of the total reducing substances: the sample (equivalent to 5 g of moisture-free tobacco), 0.5 g of $CaCO_3$, and 200 ml hot distilled water were placed in a 250 ml volumetric flask (calibrated to correct for volume occupied by the sample and the $CaCO_3$) and digested on the steam bath for one-half hour. The flask was shaken manually from time to time. The flask and contents were then cooled to room temperature, made up to volume with distilled water, mixed, and filtered. The total reducing substances in an aliquot of the filtrate were determined by the method of Bertrand (3) and calculated as glucose.

Reducing Sugars.—The sample (equivalent to 5 g of moisture-free tobacco) was extracted for 16 hours with 80% ethanol in a Soxhlet extraction apparatus; the alcoholic extract was transferred to a 250 ml volumetric flask and made up to volume with 80% ethanol. One hundred ml of the alcoholic extract was transferred to a 250 ml beaker, the alcohol was evaporated off on the steam bath and the residual aqueous solution was transferred to a 200 ml volumetric flask. The beaker was washed with several successive portions of hot water (about 80°C.) and the washings were added to the volumetric flask. The solution was cooled to room temperature, cleared with a saturated neutral lead solution, and deleaded with solid sodium oxalate as described in *Methods of Analysis* (1, p. 108). The reducing sugars in this solution (*A*) were determined by the Munson and Walker method (1, p. 506).

Sucrose.—Fifty ml of solution (*A*) was transferred to a 100 ml. volumetric flask and inverted with hydrochloric acid at room temperature, following the procedure given in *Methods of Analysis* for the determination of sucrose in grain and stockfeed (1, p. 348). The reducing sugars, after inversion, were determined as above and calculated as invert sugar. The difference between the percentage of invert sugar before inversion and the percentage of invert sugar after inversion, when multiplied by 0.95, gave the percentage of sucrose in the sample.

Dextrin.—The tobacco remaining after extraction of the sugars with 80% ethanol was dried at 100°C. to constant weight and the loss in weight due to this extraction was calculated. To 2 g of dry and extracted tobacco, 500 ml of cold distilled water was added, and the mixture was allowed to digest at room temperature for 24 hours. It was frequently shaken in the course of this digestion, and was then filtered; the residual material was washed with cold water, and the washings were

added to the filtrate. The filtrate was concentrated on the steam bath to a volume of 100 ml, 10 ml of concentrated hydrochloric acid was added, and the solution was boiled under a reflux condenser for 2 hours. The solution was then cooled, neutralized with a strong sodium hydroxide solution, cleared with a saturated neutral lead acetate solution, deleaded with solid sodium oxalate, and the reducing sugars (calculated as glucose) were determined by the Bertrand (3) method. The percentage of glucose was multiplied by 0.9 to obtain the percentage of dextrin.

Starch.—The residue from the dextrin extraction was dried at 100°C., weighed, and the starch was determined by the taka-diastase method following the procedure of Ward (44). Reducing sugars (calculated as glucose) were determined by the method of Bertrand (3). The weight of glucose found, when multiplied by 0.9, gave the weight of starch from which the percentage of starch in the original moisture-free tobacco was calculated.

Total Pectic Substances.—The percentage of pectic substances was determined by the ammonium oxalate method of Nanji and Norman (25) as modified slightly by Brückner (5, p. 347). The pectic substances were converted into calcium pectate by the Carré and Haynes (7) method, and reported in Table 1 as percentage of calcium pectate.

Pentosans.—The pectic substances were first removed by extraction with a hot 0.5% ammonium oxalate solution as above and the percentage loss in weight due to this extraction was determined. The furfural in the residual material was determined by the A.O.A.C. method (1, p. 350 developed by Tollens and his coworkers, particularly Krober (21)). The result was calculated as percentage of pentosans in the original unextracted tobacco.

Cellulose.—Percentage of cellulose was determined by the method of Kürschner and Hanak (22).

Lignin.—The sample (equivalent to 7.5 g of moisture-free tobacco) was extracted in a Soxhlet extraction apparatus first for 8 hours with 95% ethanol, and then for 4 hours with a 1:2 alcohol-benzene solution. The dried extracted material, mixed with 750 ml of a 1% hydrochloric acid solution and a few drops of capryl alcohol was boiled under a reflux condenser for 3 hours. It was then filtered on a weighed fritted glass crucible, washed with water until the washing was free of acid, dried for 4 hours in an oven at 100°C., weighed, and the combined loss due to the successive extractions was calculated. The dried and extracted material was ground, redried at 100°C. for 2 hours and a sample (0.5 to 0.8 gram) was transferred to a 50 ml Erlenmeyer flask provided with a one-hole rubber stopper through which passed a glass rod 12 cm. long with a flattened end. (The glass rod was lubricated with a drop of glycerol so that it moved easily through the hole in the rubber stopper). To the sample, 72% sulfuric acid (previously cooled to 5°C.) was added in the proportion of 5 ml of acid for every 0.1 g of sample. The reaction mixture was stirred with the glass rod, the Erlenmeyer flask was stoppered, and allowed to stand in the refrigerator (at ca 5°C.) for 24 hours with occasional stirring. The reaction mixture was then transferred to a one liter Erlenmeyer flask, sufficient distilled water was added to make a 5% sulfuric acid solution, a boiling tube about 18 cm long was put in the flask, and the mixture was boiled under a reflux condenser for 2 hours. After cooling to room temp., the crude lignin was filtered through a weighed sintered glass crucible, washed with water until free of acid, dried at 100°C. for 4 hours, and weighed. The crude lignin was ashed and the weight of ash was determined. The result was calculated as percentage of ash-free lignin in the original moisture and sand-free sample.

Methoxyl.—The percentage of methoxyl was determined by the Kirpal and Bühn modification of the Zeisel method (27). In some cases it was also determined by the semi-micro volumetric method of Vieböck and Schwappach (43). The figures in Table 1 under the heading "Methoxyl in Tobacco (Total)" represent the total

percentages of methoxyl occurring in the tobacco in ether-like and in ester-like combinations.

The percentage of methoxyl occurring in *ether-like combination* was determined as follows: the tobacco sample (equivalent to 5 g of moisture-free tobacco) plus 500 ml of 0.5% hydrochloric acid solution was boiled for one hour under a reflux condenser. The mixture was allowed to cool, filtered on a weighed filter paper, and washed with distilled water until free of hydrochloric acid. It was then dried at 100°C. to constant weight and the percentage loss due to the hydrolysis with the acid was calculated. The percentage of methoxyl in the residual tobacco was determined by the Kirpal and Bühn modification of the Zeisel method (27) and the result, calculated on the basis of the original unextracted sand-free tobacco, was recorded in Table 1 under the heading, "Ether Methoxyl in Tobacco."

The percentage of *ester methoxyl* was obtained by subtracting the percentage of ether methoxyl from the percentage of total methoxyl.

Polyphenols.—The percentage of total reducing substances (before hydrolysis) and the percentage of reducing sugars (both expressed as glucose) were determined following the procedure of Pyriki (31). The copper reduction values were determined by the method of Bertrand (3). The difference between the percentage of total reducing substances (expressed as glucose) and the reducing sugars (as glucose) gave the percentage of polyphenols (also expressed as glucose) in the sample.

Tannins.—The percentage of tannins was determined by the hide powder method, following the procedure of Brückner (5, p. 411).

Oxalic Acid.—The weighed sample (equivalent to 5 g of moisture-free tobacco) was thoroughly mixed with 20 g of acid-washed, ignited sand, 6 ml of 20% sulfuric acid, and 20 g of powdered pumice. The mixture was transferred to an extraction thimble, and extracted with ether for 24 hours in a Soxhlet extraction apparatus. The oxalic acid in the ether extract was removed by three successive 15 minute extractions with water, as suggested by Brückner (5, p. 388). The oxalic acid was precipitated as calcium oxalate following Brückner's procedure. The calcium oxalate thus obtained was dissolved in a hot dilute hydrochloric acid solution (75 ml of water and 5 ml 1:1 HCl), made neutral to phenolphthalein with sodium hydroxide solution, and then acidulated with 8–10 drops of 10% acetic acid solution. One ml of 10% calcium chloride solution was added; the solution was heated to boiling and allowed to stand overnight. The calcium oxalate obtained was ignited for 1 hour at 650°C. From the weight of CaO the percentage of oxalic acid was calculated.

Citric Acid.—The organic acids were first separated from the tobacco by exhaustive extraction with ether and the acids were extracted from the ether solution with water, following the procedure described above. The citric acid in the extract was determined by the pentabromacetone method, following the procedure of Hartmann and Hillig (16).

Laevo-Malic Acid.—The organic acids in the sample (equivalent to 5 g of moisture-free tobacco) were extracted with ether, and the ether solution was extracted with water as above. The combined aqueous extract was heated cautiously on the steam bath until the ether was removed and was then made to 100 ml. A 20 ml aliquot was transferred to a 25 ml volumetric flask, one gram of sodium acetate was added, and the solution was made alkaline to phenolphthalein by the dropwise addition of a 10% sodium hydroxide solution. The solution was made slightly acid with glacial acetic acid and one ml of this acid was added in excess. Two grams of uranyl acetate (or sufficient to saturate the solution) was added and the solution was shaken periodically in the course of 3 hours, while the flask was protected from light as much as possible. Activated carbon* (0.3 g) was then mixed in, the carbon was allowed to

* The carbon (Nuchar-C 190-N) received the following pretreatment: it was soaked overnight in a 10 per cent sulfuric acid solution; filtered; washed free of acid; dried at 75°C.; and pulverized.

settle, and the solution was made up to volume by the addition of a saturated aqueous uranyl acetate solution. It was then mixed, filtered, and the filtrate returned to the filter paper until the filtrate was bright. The *l*-malic acid in the filtrate was determined polarimetrically by the method of Dunbar and Bacon (13) as modified by Hartmann (17).

Resins and Waxes.—These were determined by the method of Pyriki (32).

Hydrogen Ion Concentration.—An aqueous extract of the ground tobacco sample was prepared following the procedure of Darkis, Dixon, and Gross (9). The *p*H of the extract was determined with the Macbeth line-operated model A *p*H meter.

RESULTS

The results on the chemical composition of the several grades of Type 11 tobacco are recorded in Table 1. It is noted that there is a considerable difference in the chemical composition among the several grades of this type with respect to a number of components. This is especially true in the case of the percentages of sand-free ash, alcohol extractives, nitrogenous constituents, total reducing substances, reducing sugars, and organic acids. The results show definitely that the several grades of this type of tobacco, which differ materially in certain physical properties that can be readily determined by a judge of tobacco from feel and visual inspection, also differ decidedly in chemical composition. The art of grading tobacco, although based entirely on subjective application of grade specifications, does bring about a segregation of the tobacco into grade units that have distinct chemical differences.

In the over-all consideration of the data recorded in Table 1, it is necessary to bear in mind that we are not primarily concerned with the absolute quantities of the several constituents of each grade. These may vary from season to season depending upon several factors, such as the seed strain, type of soil, fertilizer used, cultural practice, quantity of rainfall or other climatic conditions, the time and method of harvesting, and the curing technique. Our chief interest is in the average relative differences in chemical composition among the different U. S. grades. These differences are so great that any attempt to draw conclusions as to the chemical composition of Type 11 tobacco from analytical data obtained on samples taken at random (without regard to grade classification, as is sometimes done) would necessarily lead to misleading results.

In this connection, it must be emphasized that any deductions or conclusions presented in this paper on the relationship of chemical composition to the various quality factors and grade characteristics are based on, and limited to, the twelve grades of Type 11 flue-cured tobacco which were investigated chemically in this study. It is realized that in view of the rather limited number of grades investigated, the conclusions presented in this paper must, of necessity, be restricted in scope.

Sand and Sand-Free Ash.—The figures on the percentages of sand, included in Table 1, show the extent of contamination of certain grades

with soil material and have no other special significance. As would be expected, those grades normally coming from the lower parts of the stalks, for example, P5L, X5L, X3L, and C5L, contained fairly high percentages of sand. The B and H groups of grades contained relatively small quantities of sand (from about 0.5 to 1.5 per cent).

The sand-free ash was also much greater in the more mature, thin-bodied grades such as C5L, X3L, X5L, and P5L, as compared with the H and B groups of grades.

Petroleum Ether, Ether, and Alcohol Extractives.—Low-boiling petroleum ether is a fairly selective solvent and it extracts from tobacco mostly fatty and resinous materials, paraffin hydrocarbons, and some of the essential oil constituents. In the L colored grades, the percentage of petroleum ether extractives was low in the B group of grades, increased in the H and C groups, and then decreased in the X and P groups of grades. In the R colored grades, the percentages of petroleum ether extractives in B3R and B5GR were somewhat greater than in B3L and B5L. However, the percentage of petroleum ether extractives in H5R was much greater than in H5L.

The percentages of ether extractives were found to range from 1.66 for P5L to 3.14 for C5L. The somewhat lower percentage of ether extractives in the case of P5L was probably due to the relatively high percentage of inorganic components in this tobacco. The percentages of ether extractives in all the other grades, except C5L, were found to be of the same general order of magnitude.

Alcohol extracts a heterogeneous group of substances, among them sugars, acids, pigments, and resins. In the case of flue-cured tobacco, normally having a high percentage of sugars, the alcohol extracts would be expected to be especially rich in carbohydrate material. The data, in the main, bear this out, since the percentages of alcohol extractives were generally high in those tobacco grades having a high sugar content. In this connection it may be pointed out that in the entire L colored groups there was a regular decrease in the percentages of alcohol extractives in the fifth quality, as compared with the third quality of any pair of closely related grades. Thus the percentage of alcohol extractives in B3L was 45.77 and in B5L 45.23; in H3L 45.44, and in H5L 42.75; in C3L 42.22, and 35.04 in C5L. Similarly, the percentage of alcohol extractives in X3L was 33.93 as compared with 26.09 in X5L, while in P5L, which can be considered as a lower quality of X5L, it was 21.65. In case of the R colored group of grades, the percentages of alcohol extractives were found to be considerably lower than those in the corresponding grades of the L colored group.

The R colored group of grades differ from the corresponding L colored grades not only as to color, but also in chemical composition. This is evident not only from the percentages of alcohol extractives in these

grades, but also from a consideration of other data. B3R, B5GR, and H5R, although used to a very limited extent in the manufacture of cigarettes, are essentially non-cigarette tobaccos, whereas the L colored grades listed in Table 1 are all cigarette tobaccos.

Nitrogenous Constituents.—Considerable variation was found in the total nitrogen, protein, and nicotine content of the several grades of tobacco examined. The percentages of total nitrogen ranged from 1.92 for C3L to 3.14 for B5GR. Similarly, the percentages of proteins ranged from 5.53 for C3L to 7.97 for B5GR. It may be noted that C3L, which had the lowest percentage of total nitrogen, also had the lowest percentage of protein, and B5GR, which had the highest percentage of total nitrogen, also had the highest percentage of protein.

In general, those tobacco grades having a high percentage of total nitrogen also had a high percentage of proteins. Both the percentages of nitrogen and of protein generally varied inversely with quality, that is, the grades of fifth quality of each group contained greater percentages of these constituents than the corresponding third quality of the group. The only exception was B5L, which contained slightly lower percentages of nitrogen and protein than B3L.

The percentages of nicotine ranged from 1.52 for P5L to 4.75 for B3R, which is approximately a three-fold variation. It is rather significant that the darker-colored, heavier-bodied tobaccos, namely, B3R, B5GR, and H5R, contained much greater percentages of nicotine than did the light-bodied tobaccos of lighter shades of color. Attention is called to the fact that the R colored tobaccos of the B and H groups are also quite different in chemical composition from the L colored tobaccos of these groups with respect to the content of the various nitrogenous constituents. B3R, B5GR, and H5R were all found to contain much greater percentages of nitrogen, protein, and nicotine than the L colored grades of these groups.

Total Reducing Substances and Sugars.—The percentages of total reducing substances, which consist largely of reducing sugars in addition to a relatively small amount of other compounds and complexes capable of reducing Fehling's solution or a similar alkaline copper solution, ranged from 3.6 for P5L to 26.1 for H3L, which is approximately a sevenfold variation. In the case of the percentages of reducing sugars there was even a greater variation, ranging from 2.2 for P5L to 22.9 for H3L, or approximately a tenfold variation. It may be noted that those grades which had high percentages of reducing sugars, such as B3L, B5L, H3L, H5L, and C3L, had relatively smaller percentages of total nitrogen and proteins as compared with the grades C5L, X3L, X5L, P5L, B3R, B5GR, and H5R. Shmuk (36) who worked with Russian cigarette types, and Darkis, *et al.* (9, 10, 11, 12), in connection with their studies of American flue-cured and Turkish tobaccos, noted a similar relationship between the percentages of sugars and proteins.

The data in Table 1 show that the percentages of reducing sugars vary

directly in each group with the quality of the L colored grades. For example, the percentages of reducing sugars in B3L, H3L, C3L, and X3L were 21.5, 22.9, 20.4, and 11.1, respectively, whereas in the case of the fifth quality of these grades, namely, B5L, H5L, C5L, and X5L, it was 18.5, 20.7, 14.5, and 5.1, respectively. In P5L, which is a low subgrade of X5L, the percentage of reducing sugars was 2.2. All the R colored grades were found to have a much lower content of reducing sugars than the corresponding L colored grades.

The percentages of sucrose in all the grades were low, ranging from 0 in H5R to 1.9 in B3L and B5L.

After removal from the curing barn, flue-cured tobacco is customarily stored in a pack house in the form of large piles or bulks, wherein the tobacco undergoes certain chemical or biochemical changes. Bacon, Wenger, and Bullock (2) have shown that during this treatment or storage of the tobacco, inversion of the sucrose takes place and there is a corresponding increase in the percentage of reducing sugars. This may explain why the percentages of sucrose in all the samples were rather low.

Dextrin, Starch, Pectic Substances, and Cell-Wall Constituents.—Table 1 shows that the dextrin content of all the grades examined was low—all under one per cent—and that there was no significant variation of this constituent among the several grades.

The percentages of starch ranged from 0 in the case of X5L and P5L to 4.3 in C3L. In general, those grades with high percentages of reducing sugars also contained more starch.

With the exception of H5L, grades of the fifth quality in L color had a somewhat lower starch content than the corresponding grades of the third quality. The R colored grades had a lower percentage of starch than the corresponding L colored grades.

The analytical method employed for the estimation of the total pectic substances determines all the three recognized pectic complexes, namely, protopectin, pectin, and pectic acid. The results show that while the differences in the percentages of total pectic substances among the various grades were not great, the fifth quality of each group in L color contained in every case a somewhat greater percentage than the corresponding grade of third quality.

It may be recalled that the tobacco samples used for the determination of pentosans were first extracted with a hot 0.5 per cent aqueous solution of ammonium oxalate. This operation brings about a separation of the pectic substances (which also yield furfural when distilled with 12 per cent hydrochloric acid) from the hemicelluloses. The furfural afforded when the residual tobacco from the ammonium oxalate extraction was distilled with 12 per cent hydrochloric acid was derived entirely from the hemicelluloses, that is, from the pentosan and uronic acid components of these carbohydrate complexes.

The data, in all cases except one, show that the percentages of pentosans

varied inversely with the quality of the tobacco within each group. The one exception was X5L, which contained 3.11 per cent of pentosans or 0.18 per cent less than X3L.

The percentages of cellulose, in general, followed the same pattern as the pentosans, and in every case the fifth quality of any pair of closely related L colored grades contained a greater percentage of cellulose than the corresponding grade of third quality. This was also true in the case of the R colored grades.

It is known that the lignin content of an annual plant varies directly with its age or state of maturity, the greatest percentage of lignin being found in the most mature plant (26, 28, 29). It was hoped that the lignin content of the several grades of flue-cured tobacco under investigation would give an index of their ripeness or maturity. The results show that the greatest percentages of lignin were found in H5R, X5L, and P5L, which are considered as over-ripe or very mature tobaccos. H5R, which contained the greatest percentage of lignin, was a rather coarse, brittle, and woody tobacco. The percentages of lignin in all cases, both in the L colored and R colored grades, varied inversely as the quality within each group.

No significant differences were found in the methoxyl content of the lignin isolated from the different grades of tobacco.

Methoxyl in Tobacco.—The percentages of methoxyl (—OCH_3) in the several grades represent methoxyl present in the form of methyl esters, as in the pectins, and also that combined as methyl ethers, as in lignin and in certain uronic acids. The total percentages of these two forms of methoxyl are recorded in Table 1. The data show that the percentages of total methoxyl in all the L colored grades were of the same general order of magnitude. The R colored grades showed some differences in the percentages of total methoxyl and they also contained greater percentages than the L colored grades. The highest percentage of methoxyl (1.21) was found in H5R, which also contained the highest percentage of lignin.

The percentages of ester methoxyl ranged from 0.78 for C3L and P5L to 1.00 for H5R. Shmuk and Kashirin (38) found that the ester methoxyl (calculated as methanol) in Russian cigarette tobacco varied from 0.4 to 0.9 per cent. They claimed that there was a direct relationship between the quality of cigarette tobacco and its methanol content; the better the quality of the tobacco, the greater the percentage of methanol. No such relationship was found in the flue-cured tobacco grades investigated in this study.

The percentages of ether methoxyl in all grades was rather small and ranged only from 0.12 to 0.21. It may be pointed out, however, that H5R, X5L, and P5L, which contained the greatest percentages of ether methoxyl, also had the greatest percentages of lignin. This is to be expected since lignin is the principal source of ether methoxyl.

Polyphenols and Tannins.—Polyphenols are determined by an empirical method. The difference between the percentage of total reducing substances and the percentage of reducing sugars (both determined by the method of Pyriki (31) and calculated as glucose) affords the percentage of polyphenols (also expressed as glucose). The name "polyphenols" is rather misleading as it implies that only polyhydroxy phenolic substances are determined by this method, whereas any substance other than a reducing sugar capable of reducing Fehling's solution, or a similar alkaline copper solution, would be determined as polyphenols.

The results on the polyphenols ranged from 0.3 per cent for C5L to 2.2 per cent for B3L, and there did not appear to be any definite and consistent relationship between polyphenols content and the quality characteristics within each group of the several grades of tobacco investigated.

The percentages of tannins ranged from 1.0 for P5L to 2.6 for B3L. The results indicate that there is no definite relationship between the tannin content and the properties and characteristics of the tobacco grades studied.

Oxalic, Citric, and l-Malic Acids.—The percentages of oxalic acid in both the L and R colored grades were found to vary inversely with the quality within each group. H5L, which contained slightly less oxalic acid than H3L ,was the only exception. The R colored grades contained a greater percentage of oxalic acid than the corresponding L colored grades.

Citric acid, in general, followed the same trend as oxalic acid, that is, the poorer the quality of the L colored tobacco grade within each group, the greater the percentage of citric acid. Piatnitzki (30) also found that the percentages of oxalic and citric acids in certain Russian cigarette types varied inversely with the quality.

The percentages of *l*-malic acid did not vary regularly with the quality of all the grades, although in the C and X groups, the grade of fifth quality contained a considerably greater percentage of *l*-malic acid than the corresponding grade of third quality. P5L was found to contain 7.0 per cent of *l*-malic acid, while X5L, which is of a somewhat higher quality than P5L, contained 6.2 per cent of this acid.

(In addition to oxalic, citric, and *l*-malic acids, other organic acids are undoubtedly present in the grades of this tobacco type. There was evidence of the presence of chlorogenic acid in all the grades of Type 11 tobacco which were investigated in this study. All the grades gave a positive test for chlorogenic acid by the method of Hoepfner (18), and, following the procedure of Rosenthaler (35, p. 105), caffeic acid was obtained. The percentages of chlorogenic acid as determined by the method of Slotta and Neisser (40) ranged from 3.1 to 6.8.)

Resins and Waxes.—These two constituents are generally determined together and, of these, the resins are by far the more important from the quantitative standpoint, as the waxes are only a minor component. In the

smoking of tobacco, the resins break down into volatile aromatic substances, which contribute much to the aroma of tobacco smoke. Although the total content of resins affords some measure of the potential quantity of aromatic substances present in a tobacco, it does not indicate the quality of the aroma. The results on the percentages of resins and waxes ranged from 8.85 for B3L to 11.07 for H5R, and there did not appear to be any definite relationship between the quality of the different grades within each group and the content of resins and waxes.

From the results obtained it would appear that there is no definite relationship between the *p*H and the quality of tobacco grades investigated.

DISCUSSION*

In the over-all consideration of the analytical data presented in Table 1, and viewed from the standpoint of their relationship to the quality of the grades within each group, the various constituents listed in the table may be divided into three classes: (1) Those constituents which show a direct relationship between content and quality. (2) Those constituents which show an inverse relationship between content and quality. (3) Those constituents which apparently show no definite relationship between content and quality.

(1). In the first class may be included alcohol extractives, total reducing substances, total sugars, reducing sugars, and starch. However, it should be pointed out that since reducing sugars make up by far the largest proportion or fraction of the alcohol extractives, total reducing substances, and total sugars, the effect of the three last-named groups of constituents is due, for the most part, to reducing sugars.

(2). Among the substances belonging to the second class, the following may be included: total nitrogen, proteins, total pectic substances, pentosans, cellulose, lignin, and oxalic and citric acids. Nicotine may be considered as belonging to this class in a rather limited degree only. Although no significant differences were found in the percentages of nicotine between the third and fifth qualities of the L colored grades, considerable differences in the nicotine contents were found between the L colored grades on the one hand, and the R colored grades on the other. The R colored grades, which as a class are considered as low-quality cigarette tobaccos, contained much greater percentages of nicotine than did the L colored grades. It is realized that in the case of nicotine (and this may perhaps be equally applicable to certain other tobacco constituents), a too high content may be as undesirable from the standpoint of quality as a too low content. It is conceivable that tobacco of best quality may contain an optimum amount of nicotine, especially in relation to other tobacco constituents.

* The interpretations and opinions expressed in the following paragraphs are those of the authors and are not intended to represent the combined opinions of the Division's staff.

(3). The constituents, fractions, or complexes belonging to the third class are: petroleum ether extractives, ether extractives, methoxyl (ester and ether), polyphenols, tannins, *l*-malic acid, and resins and waxes. Acidity reported as *p*H is also apparently not definitely related to the quality of the grades within each group.

Relationship of Various "Coefficients" or "Numbers" to Tobacco Grades.—Shmuk (36) in 1924 showed that the quality of Russian cigarette tobacco varied directly with the percentage of sugars, and inversely with the percentage of proteins. This ratio:

$$\frac{\text{Per cent reducing sugars (as glucose)}}{\text{Per cent proteins}},$$

has come to be known as the "Shmuk Number" or "Shmuk Coefficient." The quality of Russian cigarette tobacco is said to vary directly with the Shmuk Coefficient and that the greater the numerical value of this coefficient, the better the quality of the tobacco. Several modifications for calculating the Shmuk Coefficient have been proposed by Kovalenko (20) and others (39). Kovalenko substituted the percentage of total nitrogen for the percentage of protein in the Shmuk formula and this ratio:

$$\frac{\text{Per cent reducing sugars (as glucose)}}{\text{Per cent total nitrogen}}$$

is sometimes referred to as the "Kovalenko Coefficient."

In 1927, Shmuk (37) presented data from which he concluded that the quality of Russian cigarette tobacco is related to the ratio of the percentage of polyphenols to the percentage of total reducing substances. This ratio:

$$\frac{\text{Per cent polyphenols (as glucose)}}{\text{Per cent total reducing substances (as glucose)}},$$

when multiplied by 100, is generally referred to in the literature as the "Polyphenol Coefficient." The quality of tobacco (Russian cigarette types) is said to vary inversely as the Polyphenol Coefficient, that is, the greater the numerical value of this coefficient, the poorer the quality of the tobacco.

In addition to Shmuk and his co-workers, who have done the pioneering work in endeavoring to correlate chemical composition of cigarette tobacco with quality as determined by the subjective methods of tobacco judges, others who have worked in this field are Rieser (34), Gärtner (15), Brückner (5), and Pyriki (33).

Brückner considers that certain constituents have a positive value, that is, they improve the quality of the tobacco, while others have a

negative value and lower the quality of tobacco. He has proposed a rather involved formula for calculating what he calls the "Quality Number" ("*Qualitätszahl*") (5, p. 298). This is obtained by dividing the sum of the percentages of sugars, starch, oxalic acid, tannins, and resins by the sum of the pH value and the percentages of pectic substances, pentosans, cellulose, lignin, ash, citric acid, total nitrogen, protein nitrogen, and nicotine, and multiplying the result by 400.

Pyriki (33) has proposed a simpler method for calculating the "Quality Number" of Turkish and related types of cigarette tobaccos. Pyriki's "Quality Number" is obtained by dividing the sum of the percentages of total reducing substances and of resins and waxes, by the sum of the percentages of nicotine, total nitrogen (less nicotine nitrogen), and total ash, and multiplying the result by 400. As in the case of the Shmuk Coefficient, the quality of the tobacco is said to vary directly with the numerical value of the "Quality Number," that is, the greater this number the better the quality of the tobacco.

In view of the fact that results of the present study indicate that the quality of the tobacco grades within each group varied directly with the percentage of reducing sugars (or total sugars) and inversely with the percentages of oxalic and citric acids, we have calculated the ratios

$$\frac{\text{Per cent reducing sugars (as glucose)}}{\text{Per cent oxalic acid} + \text{per cent citric acid}}$$

of all the tobacco grades analyzed. The numerical value of this proposed ratio would be expected to vary directly with the quality of the tobacco, that is, the greater the number, the better the quality of the tobacco within each group. The ratios of reducing sugars and organic acids are shown in Table 2, which also includes the Shmuk, Kovalenko, and Polyphenol Coefficients, and the Pyriki Quality Numbers. The ratios of reducing sugars to oxalic plus citric acids were computed from data calculated on moisture-free and sand-free bases, while the other "Coefficients" or "Numbers," in conformity with the procedures of Shmuk, Kovalenko, and Pyriki, were computed from the requisite data in Table 1, recalculated on a moisture-free basis.

In Table 2, the L colored grades were treated as a separate and distinct block from the R colored grades. The chemical composition of the R colored tobaccos is so definitely distinct from the L colored tobacco that it seemed best to treat it separately. For this reason, the three R colored grades were set off in a separate block.

In examining Table 2, it must be remembered that the quality of tobacco is supposed to vary inversely with the Polyphenol Coefficient, that is, the lower the numerical value of the coefficient, the better the quality of the tobacco, while the reverse is supposed to be true with respect to all the other coefficients, numbers, and ratios listed in the table.

TABLE 2.—*Shmuk, Kovalenko, and Polyphenol coefficients, Pyriki quality numbers and ratios of reducing sugars to sum of oxalic and citric acids of several grades of flue-cured tobacco, Type 11**

U. S. GRADE	SHMUK COEFFICIENT (1)	SHMUK COEFFICIENT (2)	SHMUK COEFFICIENT (3)	SHMUK COEFFICIENT (4)	KOVALENKO COEFFICIENT (5)	POLYPHENOL COEFFICIENT (6)	PYRIKI QUALITY NUMBER (7)	PROPOSED RATIO (8)
B3L	4.2	3.6	16.0	13.9	11.0	9.0	900	10.9
B5L	3.8	3.2	14.0	11.7	9.6	8.7	888	7.6
H3L	4.6	4.1	16.9	14.9	11.8	4.7	908	11.2
H5L	4.0	3.4	15.1	12.8	10.5	7.5	896	10.2
C3L	4.2	3.7	15.1	13.4	10.6	5.8	720	10.4
C5L	2.5	2.3	8.6	8.1	6.7	2.1	412	6.1
X3L	2.2	1.8	7.6	6.0	5.0	7.9	323	3.4
X5L	0.9	0.7	3.0	2.4	2.0	17.5	187	0.9
P5L	0.5	0.3	1.5	0.9	0.9	21.9	140	0.3
B3R	2.0	1.7	6.0	5.1	3.7	8.3	500	4.0
B5GR	1.6	1.4	5.0	4.3	3.5	9.6	536	4.2
H5R	1.3	1.0	4.6	3.6	2.8	11.0	488	2.3

(1) $\frac{\text{\% Total Reducing Substances (expressed as glucose)}}{\text{\% Proteins}}$

(2) $\frac{\text{\% Reducing Sugars (expressed as glucose)}}{\text{\% Proteins}}$

(3) $\frac{\text{\% Total Reducing Substances (expressed as glucose)}}{\text{\% Total Nitrogen—\% Nicotine Nitrogen}}$

(4) $\frac{\text{\% Reducing Sugars (expressed as glucose)}}{\text{\% Total Nitrogen—\% Nicotine Nitrogen}}$

(5) $\frac{\text{\% Reducing Sugars (expressed as glucose)}}{\text{\% Total Nitrogen}}$

(6) $\frac{\text{\% Polyphenols (expressed as glucose)} \times 100}{\text{\% Total Reducing Substances (expressed as glucose)}}$

(7) $\frac{\text{\% Total Reducing Substances (expressed as glucose)} + \text{\% Resins and Waxes} \times 400}{\text{\% Nicotine} + (\text{\% Total N} - \text{\% Nicotine N}) + \text{\% Total Ash}}$

(8) $\frac{\text{\% Reducing Sugars (expressed as glucose)}}{\text{\% Oxalic} + \text{\% Citric Acids}}$

* Shmuk, Kovalenko, and Polyphenol Coefficients, and Pyriki Quality Numbers were computed from data of Table 1 recalculated on moisture-free basis. The ratio $\frac{\text{\% Reducing sugars}}{\text{\% Oxalic} + \text{\% Citric Acids}}$ was calculated from applicable data computed on moisture-free and sand-free bases.

It may be observed that there is a very good agreement (with respect to the relative quality values within each group of the L colored grades) among the Shmuk Coefficients (calculated by four different methods), the Kovalenko Coefficients, Pyriki Quality Numbers, and with the ratios of the percentages of reducing sugars to the sum of the percentages of oxalic and citric acids. However, the R colored grades show no definite relationship to the L colored grades of the same group and quality. It is also necessary to point out, however, that when the Pyriki Quality Numbers of the L colored grades are arranged in what is supposed to be a descending order of qualities, that is, beginning with 908 for H3L and ending with 140 for P5L, that there are some inconsistencies in this relative order of qualities of the grades when compared with the coefficients and ratios (1), (2), (3), (4), (5), and (8), similarly arranged in a descending order of qualities. It is not known whether these coefficients or ratios could be applied in the classification within each group of still other grades of Type 11 or of the grades of other flue-cured tobacco types, but from the results obtained it would appear that the subject merits further investigation.

From the results in Table 2 it can be seen that there are some inconsistencies in the arrangement of the L colored group of grades according to the Polyphenol Coefficients with respect to their relative order of qualities within each group. Thus, according to the Polyphenol Coefficients, B5L and C5L are of a higher order of qualities than B3L and C3L respectively. Moreover, when the L colored grades are arranged in a descending order of qualities, that is, beginning with 2.1 for C5L and ending with 21.9 for P5L, there are some inconsistencies in this relative order of qualities of the grades, when compared with all the other coefficients and ratios given in Table 2, and similarly arranged in a descending order of qualities.

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SUMMARY

The percentages of the following constituents (all calculated on a moisture-free and sand-free basis) of twelve grades of Type 11 tobacco from which the midribs had been removed were determined: ash, petroleum ether, ether and alcohol extractives, total nitrogen, protein, nicotine,

total reducing substances, reducing sugars, sucrose, dextrin, starch, pectic substances, pentosans, cellulose, lignin, methoxyl in lignin and ether and ester methoxyl, polyphenols, tannins, oxalic, citric and *l*-malic acids, and resins and waxes. The relationships between the several constituents and the quality of the grades within a group, as determined by the subjective methods of tobacco judges, are pointed out. It is shown that the quality within each group of the L colored grades appears to be directly related to the ratio of the percentage of reducing sugars to the sum of the percentages of oxalic and citric acids.

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THE SAMPLING OF CHEDDAR CHEESE FOR ROUTINE ANALYSES*

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Cheddar cheese is made in many different styles which commonly weigh from two to 300 pounds. The cheddar style which weighs approximately 75 pounds is one of the most important commercially. It is bought and sold on the Wisconsin Cheese Exchange each week and is the style most commonly used for export and military purposes; it is usually sold under agreements which require a knowledge of moisture content.

This report discusses the problem of obtaining representative samples of the cheddar style of cheese for analysis. Although the study is con-

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cerned primarily with measuring moisture, it is obvious that analyses for fat must use the same samples.

*Official and Tentative Methods of Analysis** directs that a wedge be cut from the cheese for a sample or, if necessary, one plug or three plugs, properly drawn, may provide the sample. The rind of the plugs is rejected except when measuring absolute fat content. These directions indicate that factors such as commercial feasibility must also be considered in determining the nature of the sampling procedure.

It is common practice in industry to draw plugs with a cheese trier from any convenient spot on the flat surface exposed when the cover is lifted from a box of cheese. One or two plugs are usually taken from this exposed surface; occasionally plugs are drawn from both flat surfaces of a cheddar. More than one cheddar in the vat lot is rarely plugged. (In this discussion the term "vat lot" designates all the cheese of any style made in a vat from the same milk and by the same series of operations.)

It is common knowledge that the moisture content of a vat lot of cheddars may vary from cheese to cheese. It is even conceded by most operators that the moisture content may be slightly different in the ends of the cheddars if the cheese has been held in a box without turning. There is no general agreement on the extent of the variations to be expected, although the methods of sampling commonly used would suggest that the variations would not be important in measuring the moisture content of a vat lot of cheese.

METHODS OF ANALYSIS

In this laboratory all cheese samples were analyzed in duplicate.† Three to four gram samples, weighed on analytical balances, were dried in 50 ml Pyrex beakers without covers. Beakers and dried samples were cooled to room temperature in a desiccator before all weighings.

Samples were dried for 16 hours, to "constant weight," in a forced-draft oven operating at 110°C. This drying procedure was adopted for several reasons. It gave satisfactory agreement on current make cheese with the official A.O.A.C. method. The experimental studies demanded identical drying treatments for the samples being compared; this often necessitated drying betweeen 100 and 200 samples at the same time in the same oven. The forced-draft oven provided this capacity. This type of oven is commonly used in warehouses and laboratories where the results of this study should be of considerable interest. Finally, the satisfactory reproducibility of this drying treatment is indicated by the mean of differences between duplicate tests on each of 30 samples of 0.060 per cent and a standard deviation of ±0.045 per cent.

* *Official and Tentative Methods of Analysis of the Association of Official Agricultural Chemists*, Sixth Edition, 1945, page 336. (Seventh Edition, page 262).
† By Virginia Helmke and William C. Winder.

Other methods of their own choosing were used by the analysts in four commercial laboratories who cooperated in the last phases of this study.

SAMPLING EXPERIMENTS

Comparisons of wedge and plug samples.—The official method of sampling was followed closely, but not exactly, in a study of the moisture content of cheddar cheese. A 75-pound cheddar four weeks of age which had been paraffined for approximately 20 days was selected. The cheddar was carefully measured and marked for cutting in half horizontally. Each flat surface was also marked for cutting into 10 wedges of equal size.

Plugs were first drawn from each of the wedges. The first was drawn from the center of the flat surface and was common to all of the 10 wedges in that surface. The second plug was centered at a point one inch from the circumference, and the third was drawn at a point halfway between the other two plugs. All plugs were bored perpendicularly in respect to the flat surfaces and extended halfway through the cheese. The cheddar was next cut in half, horizontally, and each half was subdivided into the ten wedges.

Plugs and wedges were wrapped separately in heat-sealing aluminum foil as soon as they were exposed to the air and were tightly sealed. All samples were held at approximately 60°F. until analyzed. Plugs and wedges were analyzed individually.

Three-quarters of an inch of the rind end of each plug was excluded from the sample to duplicate the portion of the plug required to close the trier hole. These ends, however, were not replaced. One-eighth of an inch of "inedible" rind was removed from each wedge and the remainder was ground, thoroughly mixed, and then analyzed for moisture. Every possible precaution was taken at all times to minimize exposures which might cause changes in moisture in the samples.

Figure 1 illustrates the variations in moisture in the 20 separate wedges and in the three plugs taken from each of the wedges. The plugs overestimated the moisture content of the wedges in every section, both top and bottom, of the cheese. Differences in moisture between the top and bottom of the cheese were clearly revealed by both plug and wedge samples. The results of this experiment have been verified repeatedly in analyses of cheddars and other styles of cheese.

Comparisons of plug samples with composite samples taken from comminuted cheese.—A 3-day old vat lot of 10 cheddars was studied. The cheddars were marked in quarters and three plugs were taken from each quarter as described in the preceding experiment. Each plug was wrapped and sealed in aluminum foil as soon as it was drawn. Three-fourths of an inch of the rind end was excluded from the sample in preparing the plug for analysis.

Bandages and inedible portions were then removed from each cheddar

and the edible cheese was quickly comminuted in a large food grinder. This operation took 4 to 6 minutes. A composite sample of each cheese was collected methodically as it left the grinder. Every possible precaution was taken to minimize evaporation losses during the grinding and sampling operations.

The average percentage of moisture in the 10 ground cheddars was 37.89 per cent. The moisture in the plugs removed from the top (the small end) of the cheddars averaged 38.65 per cent, while those from the bottom

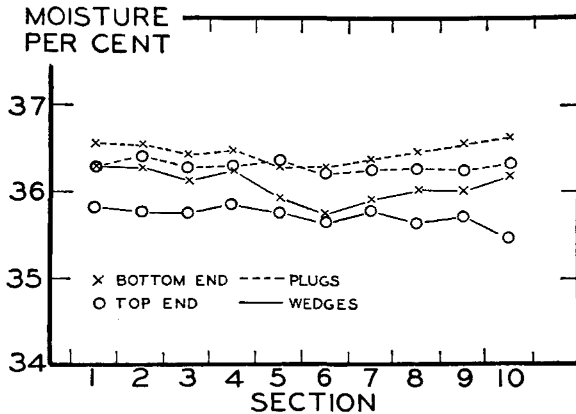

FIG. 1.—Analyses obtained from 20 wedge sections, 10 from the top half and 10 from the bottom half of a 75-pound cheddar. Three plugs were taken from each wedge for each plug sample; the remainder of each wedge was ground and mixed together for the wedge sample.

(large end) of the 10 cheddars averaged 38.78 per cent. *No composite sample from any comminuted cheddar contained as much moisture as the minimum amount found in any single plug taken from either the top or bottom of any cheddar.*

The moisture content determined by analyzing a sample of the comminuted edible portion of each cheddar must be regarded as the "true" moisture content of that cheddar. Removing plugs from the cheddar to be analyzed must be accepted as the practical method of obtaining a working knowledge of its composition when the analysis of the sample is based upon a drying procedure.

Combining plugs versus averaging analyses.—It is necessary to consider the possible effect upon the final results of this study of averaging analyses of individual plugs rather than mixing individual plugs together and then analyzing them.

Ten trials were made to compare these two procedures. Each trial was made with three plugs drawn from different parts of the same cheese.

All three plugs were practically identical in volume. Each plug was analyzed in duplicate, then the remainders of the three were mixed together in a single sample jar, and the mixture was tested in duplicate.

In these ten trials the averages of analyses of the three individual plugs differed from those of their mixtures by 0.041 per cent. The standard deviation of the differences observed was ±0.061 per cent. Such differences are not statistically significant.

Differences between duplicate tests were calculated to detect differences in homogeneity of the samples analyzed. Differences between the duplicate tests of the 30 individual plugs averaged 0.060 per cent with a standard deviation of ±0.045 per cent; the average of differences between duplicate tests on the 10 mixtures of three plugs was 0.077 per cent with a standard deviation of ±0.044 per cent. Again, the differences are statistically insignificant.

It is apparent that, for all practical purposes, averages of analyses of individual plugs are identical to analyses obtained from actual mixtures of the plugs themselves.

Sampling of 8 commercial lots of cheese.*—Eight vat lots of cheese containing a total of 54 cheddars were sampled by plugging. The lots ranged in age from 4 to 14 days, the common age of analysis when cheese is sold on the moisture basis. Three of these lots had been paraffined at the time of sampling.

Three plugs were taken from each flat surface of each cheddar. General recommendations of the A.O.A.C. were followed in locating the points of plugging. The plug on each surface nearest the outer edge was centered at one inch from the edge. Plugs on opposite surfaces were diametrically opposite each other; each extended perpendicularly halfway through the cheese. A 12-inch trier was used to obtain these plugs; it varied in diameter from $\frac{5}{8}$ inch at the tip to $\frac{6}{8}$ inch at the handle end.

In addition to these plugs, two more were drawn to simulate the usual samples taken commercially. One of these plugs was drawn from the top and the other from the bottom of each cheddar. A 5-inch trier was used for these samples; it tapered from $\frac{5}{8}$ to 11/16 inch in diameter.

One inch of the rind end of every plug was returned to the cheddar to close the hole made by the trier. Each plug was analyzed separately for moisture content. The locations and letters used to identify the 8 plugs drawn from each cheddar are shown in Figure 2.

It has been shown that representative samples must contain plugs taken from both flat surfaces of cheddars. The sampling pattern just described affords an opportunity to study the merits of several combinations of plugs. Three samples were made by combining plugs *TA1* with *A1*, *TA2*

* The managements of Lakeshire-Marty Cheese Company, Monroe, Wisconsin, L. D. Schreiber Company, Green Bay, Wisconsin, and the Kraft Foods Company, Freeport, Illinois, gave us every possible assistance in obtaining these samples.

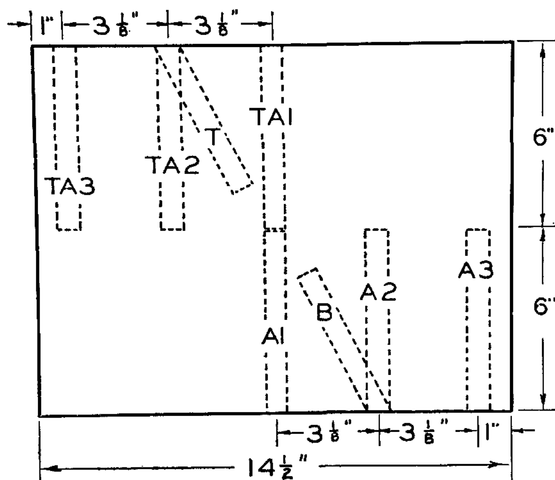

FIG. 2.—Cross section diagram of a 75-pound cheddar showing location and naming of plugs.

with *A2*, and *TA3* with *A3;* these will be designated in the discussion as *TA1A1*, *TA2A2*, and *TA3A3*. The term "combining" is used here to indicate that analyses of individual plugs were averaged. The fourth sample was made by combining all plugs of the *A* series; this six plug sample will be referred to as *TAA*. Plugs *T* and *B*, taken as shown in Figure 2, were analyzed individually, and in combination, *TB*. These plugs and their combinations thus provided a total of seven samples from each of 54 cheddars in 8 vat lots.

Table 1 shows how these chosen samples from 54 cheddars in 8 vat lots

TABLE 1.—*The average percentages of moisture in 8 vat lots of cheddars when measured by seven different samples*

VAT LOT	NUMBER OF CHEDDARS	TA1A1	TA2A2	TA3A3	TAA	T	B	TB
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
I	11	38.113	38.215	38.466	38.264	38.105	38.095	38.100
II	6	36.168	36.191	36.241	36.200	36.407	35.938	36.172
III	7	36.934	36.977	37.099	37.003	37.160	36.867	37.014
IV	5	37.683	37.797	37.201	37.560	37.438	37.944	37.691
V	5	36.028	36.178	35.901	36.036	36.358	36.028	36.193
VI	5	37.392	37.359	37.346	37.366	37.430	37.214	37.322
VII	5	36.140	36.117	36.266	36.175	36.164	36.076	36.120
VIII	10	38.690	38.688	38.900	38.759	38.574	38.806	38.690

agree in estimating the moisture content of each of the 8 vat lots of cheddars. The averages tabulated in Table 1 are practically identical. If there were any differences in the abilities of the samples to estimate the composition of the vat lot of cheddars, they are not revealed in a convincing manner in this table.

The 6-plug sample, *TAA*, approximated most closely the sample recommended for official use and should be excellent for comparison with the other less destructive samples.

Table 2 shows comparisons of analyses of the 6-plug *TAA* samples from each of the 54 cheddars with each of the 2-plug samples taken from identical cheddars. The means of the differences were small. The 2-plug, *TA1A1*, sample was the only one which differed significantly from the destructive

TABLE 2.—*The average of differences between the TAA, six-plug sample, and 4 two-plug samples from each of the 54 cheddars in the 8 vat lots of cheese*

SAMPLES COMPARED	NUMBER OF CHEDDARS	MEAN DIFFERENCE	STANDARD DEVIATION	
		per cent	*per cent*	
(TAA)-(TA1A1)	54	0.0464	0.1363	2.508 Significant
(TAA)-(TA2A2)	54	−0.0013	0.1478	0.066 Not Significant
(TAA)-(TA3A3)	54	−0.0450	0.2216	1.490 Not Significant
(TAA)-(TB)	54	0.0305	0.2187	1.023 Not Significant

TAA sample; it had an average of 0.0464 per cent less moisture. This difference is particularly interesting because it has already been shown that the actual moisture content of the edible portion of the cheddar is always less than the moisture content of samples obtained from the same cheddar by any normal plugging procedure which excludes the rind end of the plug from the sample.

STATISTICAL ANALYSIS

The reliability of each sample and control limits.—The reliability of a system of sampling depends first upon its accuracy in estimating the true moisture content of the vat lot of cheese; this can be judged in part by the data of Tables 1 and 2. A second criterion of reliability is the variability of identical samples taken from each individual cheddar in the vat lot. This second criterion is of great practical significance because it is necessary commercially to limit any routine sampling procedure to the minimum number of cheddars in the vat lot.

In Table 1 are shown the average percentages of moisture in the 8 vat lots of cheddars as they were measured by analyses of the 7 different samples. Differences of analyses of individual samples from their respective means were calculated for each sampling procedure and for each vat lot

of cheddars. The standard deviations of the 54 differences so calculated for each of the 7 sampling procedures are shown in Table 3.

The three smallest standard deviations of Table 3, listed in the order of their increasing size, were associated with *TAA*, *TA2A2*, and *TA1A1*. The difference between the first and second was 0.0064 per cent, and between the first and third, 0.0151 per cent. The standard deviations listed for the remaining four samples were distinctly larger than these three.

Three times the standard deviations listed in Table 3 represent probable limits of deviation of individual sample measurements under the conditions of these experiments. Measurements may be expected to fall outside these limits, due to chance alone, only about 3 times in 1000 samples from similar lots of cheese. These limits have been called "Control Limits." Results of analyses of individual samples which exceed these control limits are so unusual that, when they occur, they indicate a defective sample or an influence other than chance. Cheddars which do not belong in the vat lot might be expected to yield measurements outside these control limits.

TABLE 3.—*Standard deviations and control limits of the differences between moisture in samples from individual cheddars and the mean moisture content of all similar samples from their respective vat lots. Data from 54 cheddars in 8 vat lots*

SAMPLE	STANDARD DEVIATION (σ) OF DIFFERENCES	CONTROL LIMITS (3σ)	SAMPLE	STANDARD DEVIATION (σ) OF DIFFERENCES	CONTROL LIMITS (3σ)
	per cent	*per cent*		*per cent*	*per cent*
TA1A1	±0.2164	±0.6492	T	±0.3224	±0.9672
TA2A2	±0.2077	±0.6231	B	±0.3210	±0.9630
TA3A3	±0.2952	±0.8856	TB	±0.2624	±0.7872
TAA	±0.2013	±0.6039			

The control limits of the multiple-plug samples are arranged from left to right in Table 4 in the order of their increasing variability. The least variable, or most reliable, was the *TAA*, 6-plug sample; the most variable was the *TA3A3*, 2-plug sample which contained the plugs taken from the points nearest the edge of the cheddars.

Table 4 shows how the reliability of the sample from a vat lot of cheese can be increased by taking samples from more than one cheddar in the vat lot. When the *TAA* sample was taken from one cheddar per vat, the measurement was expected to be within 0.6039 per cent above or below the mean of similar samples taken from every cheddar in the vat lot. Combining and analyzing the same plugs taken from 2 cheddars reduced the control limits to 0.4270 per cent, while combining the same plugs from 4 cheddars of the vat lot cut the control limits in half.

Comparisons of the control limits of the samples shown in Tables 3 and 4 indicate that *TA2A2* and *TA1A1* are almost as reliable as the 6-plug sample. They are much less destructive. The next best 2-plug sample is *TB* which in these trials had a control limit of ±0.7872 per cent. The single plugs *T* and *B* had control limits in excess of ±0.96 per cent; they are relatively unreliable. Combining them to form the *TB* sample still does not produce the confidence justified by the *TA1A1* and *TA2A2* samples.

Applying the control limits of the TA1A1 sample to vat lots of different sizes.—The control limit of ±0.6492 per cent for *TA1A1* has been calculated from the data obtained in analyzing the 54 cheddars of 8 vat lots.

TABLE 4.—*Control limits for deviations from their vat means of samples from one to five cheddars per vat lot.* Data from 54 cheddars in 8 vat lots*

NO. OF CHEDDARS PER SAMPLE	TAA 6-PLUG	TA2A2 2-PLUG	TA1A1 2-PLUG	TB 2-PLUG	TA3A3 2-PLUG
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
1	±.6039	±.6231	±.6492	±.7872	±.8856
2	±.4270	±.4406	±.4591	±.5566	±.6262
3	±.3487	±.3597	±.3748	±.4545	±.5113
4	±.3020	±.3116	±.3246	±.3936	±.4428
5	±.2701	±.2787	±.2903	±.3520	±.3961

* "Control limit" of samples from more than one cheddar per vat lot $=3\sigma/\sqrt{n}$; "n" is the number of cheddars per vat sampled.

This control limit is 3 times the square root of the within-vat-lot mean square. This limit must be adjusted according to the number of cheddars in a single vat lot if it is to be used in conjunction with the calculated mean of that vat lot. These adjusted control limits for vat lots of common sizes have been calculated by multiplying ±0.6492 by the square root of $(N-1)/N$, where N is the number of cheddars in the vat lot; Table 5 presents the results.

"Control limits" for moisture tests in commercial laboratories.—Control limits for moisture tests are affected by differences in technicians, methods, and equipment used. It seemed desirable to determine control limits characteristic of well-managed commercial laboratories.

Four laboratories* cooperated by taking samples, analyzing them, and sending the results to the University for study. Instructions for sampling were given to the men in charge of the actual sampling and analytical work. Each laboratory sampled and analyzed 3 vat lots of cheddars. The *TA1A1* sample, which we will call the "core" sample, was taken from every cheddar in each of these lots. Each cheddar was also sampled by the

* The laboratories which gave this most valuable assistance were: Kraft Foods Company of Wisconsin, Pauly Cheese Company, L. D. Schreiber Company, and the Wheeler Corporation, all located at Green Bay, Wisconsin.

TABLE 5.—*Control limits ($3\sigma = \pm 0.6492$) for sample TA1A1, adjusted to vat lots of different sizes. Data from 54 cheddars in 8 vat lots*

CHEDDARS IN VAT LOT (N)	CONTROL LIMIT*	CHEDDARS IN VAT LOT (N)	CONTROL LIMIT*
	per cent		*per cent*
3	±.5301	11	±.6190
4	±.5622	12	±.6216
5	±.5807	13	±.6237
6	±.5926	14	±.6256
7	±.6010	15	±.6272
8	±.6073	16	±.6286
9	±.6121	17	±.6298
10	±.6159	18	±.6309

* Adjusted control limit $= \pm 3\sigma \sqrt{(N-1)/N}$, where N = number of cheddars in the vat lot.

procedure usually followed in that laboratory. All samples were analyzed in duplicate for moisture and fat. Each laboratory used its own analytical technique.

The analyses for moisture were not reported to the same degree of accuracy by all laboratories. One reported to the nearest 0.01 per cent, another to the nearest 0.05 per cent, and two to the nearest 0.1 per cent. The data were used exactly as reported.

The results of duplicate tests for moisture in each sample were averaged. These values from each cheddar in the vat lot were then averaged to obtain the mean percentage of moisture in the lot. The deviations of the means of the duplicates from the mean of all cheddars in that vat lot were computed. This was done both with analyses of samples obtained by the core sampling method and also with those obtained by the usual sampling method employed by each cooperating laboratory. These differences were used to calculate the control limits presented in Table 6.

The control limits shown in Table 6 indicate the wisdom of calculating these limits in different laboratories. The limits associated with the core sample were practially identical in three of the four laboratories. Labora-

TABLE 6.—*The percentage moisture control limits* for deviations of individual cheddars from the means of their vat lots*

LABORATORY	NUMBER OF		CORE SAMPLE CONTROL LIMITS*	"USUAL" SAMPLE CONTROL LIMITS*
	VATS	CHEDDARS		
			per cent	*per cent*
A	3	31	±.7638	±.6354
B	3	28	±.5073	±.7356
C	3	34	±.7623	±.9753
D	3	32	±.7506	±.9060

* Control limits are not adjusted for vat size; they equal 3σ of the differences between the percentage moisture in each cheddar and the mean of all cheddars in its vat lot.

tory *B* showed a smaller control limit despite the fact that the measurements of weights were made with torsion balances; no explanation is offered. The control limits associated with the core sample taken by the three commercial laboratories were approximately 0.11 per cent larger than those resulting from measurements on commercial lots of cheese analyzed by the technicians in the laboratory at the University.

The control limits shown in Table 6 for the core sample were smaller with one exception, Laboratory *A*, than those calculated from samples taken by the method commonly used in the commercial laboratory. The exceptional laboratory, *A*, combined 3 plugs taken from each cheddar in the general locations of the *A1*, *A2*, and *A3* plugs in Figure 2. The greater magnitude of the limits calculated from the data of the other laboratories can probably be attributed to the use of the *T* or *B* plugs taken somewhat

TABLE 7.—*Percentage moisture control limits for the core sample and for three different probabilities estimated by pooling the data from laboratories A, C, and D*

NUMBER OF CHEDDARS SAMPLED IN VAT LOT	CONTROL LIMITS FOR PROBABILITY		
	.9973	.90	.80
	per cent	*per cent*	*per cent*
1	±.759	±.416	±.324
2	±.537	±.294	±.229
3	±.438	±.240	±.187
4	±.380	±.208	±.162
5	±.339	±.186	±.145

as shown in Figure 2. The general trends of effects of such sampling treatments on control limits have been shown in Table 3.

It was shown in Table 4 that increasing the number of cheddars sampled per vat decreased the control limits of the estimate of the moisture in that vat lot. Table 7 shows the comparable data obtained when the analyses from commercial laboratories *A*, *C*, and *D* were pooled in making the calculations. Under these commercial conditions analyses of the core sample from a single cheddar estimated the mean of its vat lot within control limits of ±0.759 per cent. These limits are not adjusted for number of cheddars per vat lot. The comparable control limits obtained by analyses in the laboratory at the University were ±0.649; these are the limits shown in Table 4 for the *TA1A1* sample from a single cheddar in a vat lot. These control limits are associated with a probability of 99.73 per cent.

If the limits of ±0.649 per cent and ±0.759 per cent are too great to satisfy the purposes of the analyst, then it is possible to reduce them by combining and analyzing samples from more than one cheddar per vat. Tables 4 and 7 show, for example, that a 5-cheddar core sample produced limits of ±0.29 and ±0.34 per cent in the University and commercial laboratories, respectively.

If the probability of 99.73 per cent is unnecessarily exact, then smaller control limits associated with lower probabilities may be chosen for control purposes. Table 7 shows the control limits associated in this study with probabilities of 90 and 80 per cent. The commercial laboratories sampling by the core method could expect that the percentages of moisture in 8 out of 10 cheddars in any single vat lot would differ from the mean of their vat lot by not more than ± 0.324 per cent; under the same conditions 9 out of 10 cheddars would differ from the mean of their vat lot by not more than ± 0.416 per cent. By analyzing combined core samples from two or more cheddars per vat lot, the control limits decrease for each degree of probability; these decreasing limits are shown in Table 7.

DISCUSSION

The structure of cheddar cheese consists of two clearly different portions: the rind, which varies in thickness, and the interior. The rind is the protective shell which is commonly drier than the interior, but not always so. It is edible in part but it is not usually eaten as natural cheese.

The rind removed in sampling cheese must be replaced if the cheese is to be moved subsequently into commercial channels. The rind from a wedge sample cannot be restored to protect the interior. At least one inch of the rind end of a plug sample should be returned to close the opening. The necessity of excluding this one inch of the rind from samples for analysis is extremely important because this one-inch outer layer of the cheddar style of cheese is more than one-fourth of the total volume.

Comminuting and mixing together the edible portions of the cheese, including the rind, yields a representative sample for measuring what might be called the "true" moisture content of the cheese. This "true" moisture content is not represented in a sample formed by removing plugs, restoring the rind ends of the plugs to close the holes, and using the remainder of the plugs for analysis. Despite its obvious bias, such a plug sample, if properly taken, must be regarded at present as the only one practical for commercial use; on analysis it yields what might be called a "working" knowledge of the moisture content of the cheese.

The differences to be expected in practice between the "true" and "working" moisture contents of a cheddar cheese cannot be estimated accurately since they vary with the conditions affecting rind formation. The rind area of cheese must be definitely formed by drying before the cheese is paraffined. It is at the time of paraffining that cheese is usually sampled and analyzed to determine its value as it enters commercial trade channels.

The facts presented in this study suggest that the two-plug sample, *TA1A1*, which we have termed the "core" sample, is the most practical commercial sample to use in analyzing the cheddar style of cheese. The reasons for the choice can be summarized:

The only practical method of obtaining a sample for analysis without destroying the commercial value of the cheese is to plug the cheese and use the rind end of the plugs to close the holes.

Excessive damage to the commercial value of a cheddar is caused by the removal of more than two plugs.

Analyses of samples obtained by plugging tend to overestimate the "true" moisture content of the edible portions of the cheese. The core sample tends to underestimate the moisture revealed by any other system of plugging studied.

The "control limits" shown in Table 4 for the core sample, *TA1A1*, were wider by 0.052 per cent than the *TA2A2* sample. The practical significance of this small difference does not disqualify the core sample when its other advantages are considered.

It is easy to determine the points for plugging to obtain the core sample; the plugs remove the vertical axis, or "core," of the cheddar.

The core sample causes less damage to the cheese than any other two-plug sample. When the cheddar is sold as natural cheese and cut into wedges, the trimming of the ends of the wedges eliminates all signs of the plug, if any trimming is necessary. If the cheese is quartered for processing, the plug holes are entirely exposed for inspection.

The trials of the core sample by commercial laboratories indicate that control limits disclosed by any laboratory may not be identical to those associated with the results in other laboratories. Variations in analytical techniques, equipment, and perhaps in the source of material analyzed can be expected to cause such variations.

The control limits of the commercial laboratories shown in Table 6 were slightly larger than the control limits reported in Table 4. The small variations may be attributed to the differences between routine analyses under commercial conditions and experimental analyses in a research laboratory.

In commercial laboratories operated under satisfactory conditions the control limits of Tables 7 and 8 can probably be applied with reasonable confidence when the core sample is used. Greater confidence in the analytical results in any single laboratory can only be attained by similar studies applied to the routine work of that particular laboratory.

The control limits discussed in this study are, of course, for moisture content of samples as determined. They take into account the variability attributable to analytical procedures. They cannot take into account bias. Bias has been mentioned in discussing the real difference between the moisture content of core samples and that of the whole cheddar from which they were taken. Bias may also enter from analytical procedures or from sampling cheese in various stages of ripening; either of these sources of bias might cause variations in the volatility of substances other than water during the drying of the cheese.

TABLE 8.—*Control limits ($3\sigma = \pm 0.759$) for the core sample adjusted to vat lots of different sizes. Data from 97 cheddars in 9 vat lots analyzed by laboratories A, C, and D*

CHEDDARS IN VAT LOT (N)	CONTROL LIMIT*	CHEDDARS IN VAT LOT (N)	CONTROL LIMIT*
	per cent		*per cent*
3	±.620	11	±.724
4	±.657	12	±.727
5	±.679	13	±.729
6	±.693	14	±.731
7	±.703	15	±.733
8	±.710	16	±.735
9	±.716	17	±.736
10	±.720	18	±.738

* Adjusted control limit $=3\sigma\sqrt{(N-1)/N}$; N = number of cheddars in the vat lot.

CONCLUSIONS

1. The true moisture content of the edible portion of a 75-pound cheddar cheese is always less than the moisture content of plugs taken from that cheddar when the rind end of the plug is used to close the orifice of the hole made by the trier.

2. The sample preferred for commercial analysis of a cheddar is obtained by drawing two plugs, one from the top, the other from the bottom of the cheese. Each plug extends perpendicularly halfway through the cheese and is drawn from the center of each flat surface. One inch of the rind end of each plug is used to close the orifice of the trier hole. Plugs are between $\frac{5}{8}$ and $\frac{6}{8}$ inches in diameter. This is called the "core" sample.

3. Under experimental conditions, analyses of the core sample obtained from a single cheddar in a vat lot estimated the mean percentage of moisture of similar samples taken from all the cheese in that vat lot within the control limits (3σ) of ± 0.649. Under satisfactory commercial conditions analyses of the core sample from a single cheddar estimated the mean of its vat lot within control limits of ± 0.759. These limits are not adjusted for number of cheddars per vat lot.

4. These control limits of the core sample can be reduced without loss of confidence by combining and analyzing samples taken from two or more cheddars per vat lot.

5. These control limits will vary with the technique and equipment used. They can be applied with reasonable satisfaction where conditions of analysis include the use of chemical balances, desiccators, forced draft or vacuum ovens, duplicate tests of samples, and trained technicians.

ACKNOWLEDGMENT

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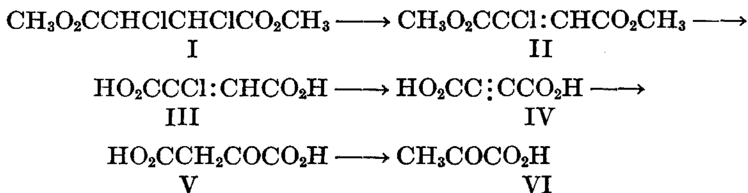
statistical phases of this study and for reviewing and criticizing the manuscript, and to the Computing Service of the University for making the calculations.

THE DETERMINATION OF DIMETHYL DICHLOROSUCCINATE

By L. L. Ramsey and W. I. Patterson (Food and Drug Administration, Federal Security Agency, Washington, D. C.)

A recent proposal to employ dimethyl dichlorosuccinate (DMDCS) as an antimycotic in packaging foods such as cheese, bread, fresh raspberries and fresh tomatoes (1) has created the need for a sensitive, specific method for the detection and determination of this compound. Since DMDCS can readily be dehydrohalogenated (2), a method might be based upon organic chlorine, but such a method would lack both sensitivity and specificity. The colorimetric method described here is based upon the potassium hydroxide degradation of DMDCS to pyruvic acid, which is then measured with 2,4-dinitrophenylhydrazine by the technic of Friedemann and Haugen (3). This method has been applied to cheese containing DMDCS and to cheese wrappers impregnated with DMDCS.

The degradation of DMDCS by potassium hydroxide proceeds in accordance with the following reactions (2, 4, 5, 6):



Number I is a mixture of the meso and racemic isomers of dimethyl dichlorosuccinate; Number II, dimethyl chlorofumarate; Number III, chlorofumaric acid; Number IV, acetylene dicarboxylic acid; Number V, oxalacetic acid; and Number VI, pyruvic acid. The reactions outlined about may not necessarily occur stepwise and in the order indicated; some may possibly occur simultaneously.

Although the hydration of an acetylenic acid with potassium hydroxide solution to produce a keto acid has long been known (6), this reaction has not to our knowledge been utilized as the basis for an analytical method. As is indicated by the above reactions, all of the intermediate compounds, if present initially in the sample extract, will be converted to pyruvic acid and be measured as DMDCS. However, since none of these compounds is known to occur naturally in cheese and since a technic quite specific for

pyruvic acid (3) is employed, the method is highly specific for DMDCS and its possible degradation products in cheese. (Control cheeses containing no added DMDCS show blank values of 0.2–0.5 p.p.m. apparent DMDCS.)

METHOD

REAGENTS

(a) *Standard solution of DMDCS.**—200 micrograms/ml. Accurately weigh 100 mg of the DMDCS in a small weighing boat and transfer to a 500 ml volumetric flask with water; wash the boat in a funnel placed in the neck of the flask with copious amounts of water as the DMDCS is not very soluble. Dilute to the mark and shake vigorously.†

(b) *Potassium hydroxide solution.*—Dissolve 75 g reagent grade KOH in water, cool and dilute to 100 ml. Adjust to 10.7 ± 0.3 *N*. Store in Pyrex or paraffin-lined bottle.

(c) *2,4-Dinitrophenylhydrazine solution.*—Triturate 100 mg reagent with 18 ml of concd HCl in a mortar with a pestle. Transfer to an Erlenmeyer flask with 82 ml of water, shake vigorously, and filter. Store in a refrigerator when not in use; if a precipitate forms, filter.

(d) *Sodium hydroxide solution.*—ca 1.5 *N*. Store in Pyrex or paraffin-lined bottle.

(e) *Sodium carbonate solution.*—Dissolve 10 g Na_2CO_3 in water and dilute to 100 ml.

PREPARATION OF THE STANDARD CURVE

Pipet 0, 0.50, 1.00, 2.00, 3.00, 4.00, and 5.00 ml aliquots of the standard DMDCS solution into 10 ml Pyrex glass-stoppered graduated cylinders. To each add 2 ml KOH solution and sufficient water to give a volume of 7 ml. Stopper the cylinders and mix. *Remove* the stoppers and place the cylinders in a boiling water bath for 1 hour, taking care to maintain the level of the water in the bath above the levels of the solutions in the graduated cylinders. At the end of the 1 hour period remove the cylinders from the bath and cool by placing in a cold water bath. To each cylinder add sufficient concd HCl to give an excess of 0.10 to 0.15 ml over the amount required for neutralization of the KOH as determined by a previous titration. Mix the solutions and cool to room temperature. Dilute each exactly to the 10 ml mark and mix again.

Determine the pyruvic acid (3) as follows: Pipet a 3 ml aliquot of solution from each cylinder into correspondingly numbered test tubes, 18 mm×150 mm or 25 mm ×175 mm. Place the test tubes in a water bath at approximately 25°C. After the tubes have tempered for 5 minutes, add 1 ml of the 2,4-dinitrophenylhydrazine reagent to the first tube, mix, and allow to react 5 minutes. Remove the reaction tube from the bath, add exactly 3 ml of toluene or benzene, and pass a rapid stream of air (or nitrogen) thru the mixture for a period of exactly 2 minutes. (The air or nitrogen is blown thru a glass tube drawn to a capillary point to mix the phases thoroughly.) After the phases have separated, remove and discard most of the aqueous layer by means of an eyedropper pipet. Swirl the tube to dislodge the aqueous solution adhering to the walls and remove the remainder of the aqueous layer. Add exactly 6 ml of the sodium carbonate solution to the tube and mix the phases by passing a rapid current of gas thru the mixture for a period of exactly 2 minutes as above.

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* A sample of technical dimethyl dichlorosuccinate (chlorine: cald'd 32.98%, found 32.89%) kindly supplied us by the manufacturer, National Aniline Division, Allied Chemical & Dye Corporation, New York, N. Y., was used as the reference standard in this work.

† DMDCS solutions several months old give the same results as freshly prepared solutions.

Allow the mixture to stand until the phases separate and the aqueous layer becomes clear. Insert a 5 ml pipet thru the upper layer to the bottom of the tube and blow just sufficient air thru the pipet to discharge the small amount of toluene or benzene which enters the pipet. Transfer 5 ml of the carbonate solution to another test tube, taking care to wipe the tip of the pipet free of adhering solvent before the volume is adjusted to the mark. Repeat this procedure for each of the 3 ml aliquots in the numbered tubes. (In order to facilitate the work, the 2,4-dinitrophenylhydrazine reagent can be added to the tubes successively at 2.5 minute intervals. As the 5 minute reaction period elapses for a given tube, the solvent is quickly added to it from a buret, and the phases are mixed for 2 minutes by the rapid stream of air. After all of the samples have been extracted with benzene and the aqueous layers removed, the benzene layer in each case is then extracted with the carbonate solution.) To each of the 5 ml sodium carbonate aliquots, add exactly 5 ml of the 1.5 N NaOH, swirl the tubes immediately to mix the contents, and using a suitable instrument and cell length, read the colors at 520 mμ 5–10 minutes after the addition of the NaOH. Read all of the tubes, including the 0 tube, against a blank of distilled water. Plot the absorbancies against the quantities of DMDCS originally placed in the 10 ml graduated cylinder.

PREPARATION OF SAMPLE

Cheese.—Weigh 50 g cheese, cut into small pieces, and transfer to a blendor placed in a hood with the draft on. Add 75 ml of petroleum ether (30–60°C.) to the cheese and comminute 2 minutes (*caution: great danger of splashing, with resulting fire*; use of a rheostat or variable transformer recommended to control speed of blendor). Place a filter paper on a 2 to 3 in. diameter sintered glass Büchner funnel, transfer the cheese mixture to the funnel by means of a spatula and filter with suction. Press the cake to remove most of the solvent. Return the cake to the blendor and repeat the extraction with another 75 ml portion of petroleum ether. Filter into the same suction flask previously used. Again return the cake to the blendor and repeat the extraction and filtration.* Transfer the petroleum ether filtrate-extracts to a 400 ml beaker and rinse the flask with 2 small portions of solvent. Place the beaker on a steam bath, and concentrate the solvent to ca 65 ml (beaker marked at 65 ml). Transfer the extract to a 125 ml separatory funnel and rinse the beaker with two 5 ml portions of solvent. Extract the petroleum ether solution with three successive 15 ml portions of acetonitrile, drawing off the extracts into a second 125 ml separatory funnel. To the combined acetonitrile extracts add 5 ml of water and 10 ml of petroleum ether, and shake vigorously. After the phases separate, draw off the acetonitrile layer into a 200 ml round bottom flask and add 2 ml of the KOH solution. Add a boiling chip, place the flask in a water bath maintained at ca 50°C. (not above 55°C.) and remove the acetonitrile *in vacuo*.

Cheese Wrappers.—If the wrapper is unused, remove paper backing and cut the wax wrapper into small pieces. Place the pieces in a blendor and extract with three successive 75 ml portions of petroleum ether, decanting the extract each time into a 400-ml beaker. Concentrate the combined extracts to ca 40 ml on a steam bath in a hood. Transfer the concentrate to a 50 ml centrifuge tube, wash the beaker with two 5 ml portions of petroleum ether, and cool to room temperature. Centrifuge and decant the petroleum ether into a 125 ml separatory funnel. Add 10 ml petroleum ether to the residue in the centrifuge tube. Shake to disperse the residue, centrifuge, and decant the wash solvent into the same funnel. Repeat the washing

* This extraction procedure was found satisfactory for all types of cheese investigated except cream and limburger. With these two types add 50–75 g of anhydrous Na_2SO_4 to the blendor with the cheese and make 4 extractions with petroleum ether instead of the 3 directed above. If the extract of limburger will not filter thru the Büchner, decant the extract thru a rapid fluted filter paper, pressing the solid material with a spatula to remove most of the solvent.

with another 10 ml portion of petroleum ether. Proceed with the acetonitrile extraction as under "Preparation of Sample, Cheese" above.

DETERMINATION

Immediately after all of the acetonitrile has been removed (absence of 2nd phase or of oily droplets) release the vacuum. Transfer the alkaline solution to a 10 ml glass-stoppered graduated cylinder with an eyedropper pipet. Use just sufficient water for the transfer (3 washings of ca 1 ml each) to give a final volume of 7 ml in the cylinder. Mix the contents and proceed with the boiling water bath treatment, the cooling, acidification, and dilution to the 10 mark as described above for the standards under "Preparation of the Standard Curve." (In diluting to the 10 ml mark, neglect the volume occupied by the small quantity of insoluble solids.) Mix thoroughly, filter thru a small folded filter, and pipet a 3 ml aliquot of the filtrate into a test tube for color development. Continue exactly as under "Preparation of the Standard Curve" beginning with the sentence: "Place the test tubes in a water bath at approximately 25°C."

EXPERIMENTAL RESULTS AND DISCUSSION

Development of the Method.—In the development of the method a study was made to determine the optimum conditions for the degradation of DMDCS to pyruvic acid. The effect of varying the KOH concentration while other factors are held constant is illustrated in Table 1. (A Beckman Model DU spectrophotometer was used in this work.) There appears to be very little, if any, difference between the results obtained with normalities ranging from 2.8 to 3.5 N KOH. Two ml of a KOH solution 10.7 ± 0.3 N diluted to 7 ml will give a normality ranging from 2.97 to 3.14, approximately the middle of the 2.8 to 3.5 N range; therefore, the 10.7 ± 0.3 N KOH was adopted. The effect of time in the boiling water bath is illustrated in Table 2. These data show that maximum yields of pyruvic acid are obtained in 1 hour, but that heating for an additional hour does not appreciably affect the results. The one hour heating period was therefore adopted. As is illustrated in the standard curve, Figure 1, the relationship between intensity of color and quantity of DMDCS ini-

TABLE 1.—*Effect of KOH concentration on the degradation of DMDCS: 500 mmg DMDCS, 7 ml reaction volume, 1 hr. in a boiling water bath. Measured at 520 mμ in 1 cm cuvette*

ML 10.4 N KOH	NORMALITY OF FINAL SOLUTION	ABSORBANCY (520 mμ)
1.00	1.48	0.280
1.50	2.23	0.405
1.75	2.60	0.445
1.90	2.82	0.485
2.00	2.97	0.486
2.10	3.12	0.487
2.25	3.34	0.465
2.33	3.46	0.488
2.50	3.71	0.450

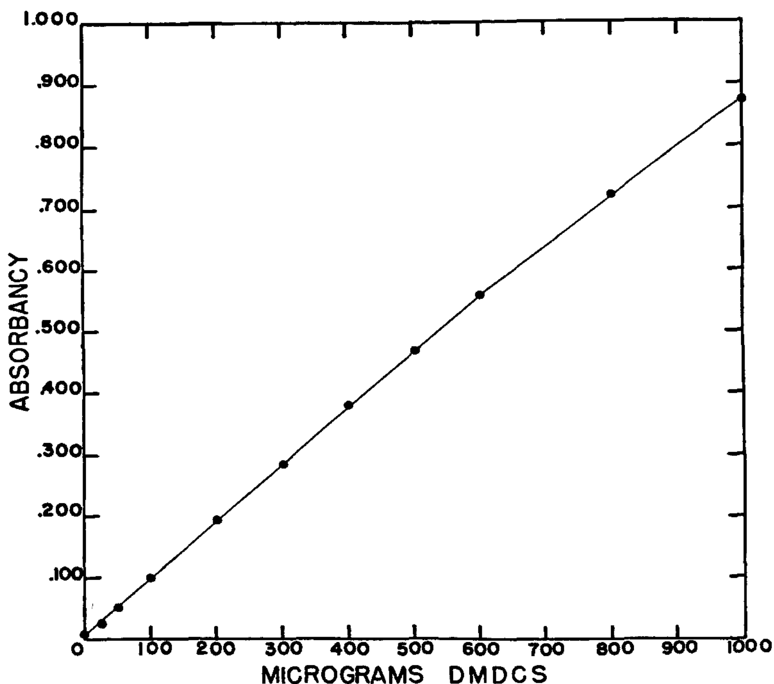

FIG. 1.—Standard curve for DMDCS. A 3/10 aliquot of the quantities of DMDCS represented in this graph were taken for the color development. Absorbancy was measured in a Beckman spectrophotometer, Model DU, at 520 mμ in a 1 cm. cuvette.

tially present in the reaction cylinder follows Beer's law over the range 25–600 micrograms DMDCS, but above 600 mmg there is a slight deviation from the straight line function.

On the basis of the data in Tables 1 and 2 and of the excellent reproducibility of the standard curve, it appeared that the maximum values for pyruvic acid might be the theoretical values. This hypothesis was tested; the data in Table 3 show the percentage conversion to pyruvic acid of DMDCS and all of its intermediate degradation products. It is noteworthy that the percentage yield of pyruvic acid obtained by the degradation of these compounds is essentially the same, viz., 72 to 78 per cent in all cases except oxalacetic acid; the decarboxylation of oxalacetic acid by the method to produce pyruvic acid is practically quantitative, 91 per cent.

The standard used in these studies was a pure sample of pyruvic acid-2,4-dinitrophenylhydrazone, m.p. 212–213°C. A solution of this hydra-

TABLE 2.—*Effect of time in the boiling water bath on the degradation of DMDCS: 500 mmg DMDCS, 7 ml reaction volume, and 2 ml 10.4 N KOH. Measured at 520 mμ in 1 cm cuvette*

MINUTES	ABSORBANCY
15	0.325
30	0.420
45	0.465
60	0.486
75	0.473
90	0.470
120	0.468

zone in alcohol containing exactly 0.6 micromol was placed in a test tube and the solvent was carefully evaporated under a gentle current of air. The residue was dissolved in 3 ml of hot water, the solution cooled to 25°C., and the colorimetric determination of pyruvic acid carried thru in accordance with the method under preparation of standard curve beginning with "Place the test tubes in a water bath at approximately 25°C." Millimolar solutions of each of the other compounds in water were prepared and 2 ml of each (2 micromols) transferred to the 10 ml glass stoppered reaction cylinders. Two ml of the KOH reagent was added, the solution diluted to 7 ml, and the determination carried thru in accordance with the method (color developed on 3 ml aliquot of the 10 ml or 0.6 micromol of the compound). The DMDCS used was the technical product referred to above. The chloromaleic acid was prepared from chloromaleic anhydride (Eastman practical grade) and a portion of the acid was esterified with methanol-H_2SO_4 to obtain the dimethyl ester. After two crystallizations

TABLE 3.—*Conversion of DMDCS and its derived products to pyruvic acid. 0.6 micromol of each compound in the 3 ml aliquot taken for color development. Absorbancy measured at 520 mμ in 1 cm cuvette*

COMPOUND	ABSORBANCY	PER CENT CONVERSION TO PYRUVIC ACID
Pyruvic acid—2,4-dinitrophenylhydrazone (Standard)	0.570	100
DMDCS	0.415	72
Dimethyl chlorofumarate	0.432	75
Chlorofumaric acid	0.420	73
Dimethyl chloromaleate	0.432	75
Chloromaleic acid	0.450	78
Acetylene dicarboxylic acid	0.418	73
Oxalacetic acid*	0.522	91

* Obtained from Mann Fine Chemicals, Inc., New York, N. Y.

from acetic acid-chloroform the acid melted at 107–108°C. and the ester distilled at 79–80°C. at 5–6 mm. The chlorofumaric acid was prepared by hydrolysis of the DMDCS with concd HCl and evaporation of the HCl on the steam bath (5, 7). A portion of the acid was crystallized twice from acetic acid, after which it melted at 194°C. Another portion was esterified with methanol-sulfuric acid to obtain the dimethyl ester, which distilled at 96–97°C. at 7 mm. The acetylene dicarboxylic acid was prepared from DMDCS (2).

Reagent blanks were studied and found to be very low. Three ml of distilled water was treated with 1 ml of the dinitrophenylhydrazine reagent, extracted with toluene, the toluene extracted with the Na_2CO_3 reagent, and the color developed with the NaOH reagent; this blank read 0.005 against distilled water. A blank on 5 ml distilled water plus 2 ml of the KOH reagent was carried through the method in duplicate; the readings against distilled water were 0.006 and 0.008. When 35 ml acetonitrile and 100 ml of petroleum ether were included in the blank, the reading against distilled water was 0.010.

Application of the Method to Cheese.—In applying the method to cheese the separation of the DMDCS from fat was a problem. Acetonitrile, previously found useful for separating many insecticides from fat and waxes and other interfering material in plant and animal tissue by Jones and Riddick (8), was also found satisfactory for DMDCS. An experiment was performed to determine the distribution of DMDCS between petroleum ether and acetonitrile when 20 ml of acetonitrile and 20 ml of petroleum ether (30–60°C.) containing 1 mg of DMDCS were equilibrated in a separatory funnel; 4 per cent of the DMDCS was found in the petroleum ether. Three successive 15 ml extractions with acetonitrile from 75 ml petroleum ether containing 1 mg DMDCS was found to extract the DMDCS quantitatively. When the 75 ml of petroleum ether contained about 15 g fat from a 50 g sample of cheese, some fat was extracted by the acetonitrile. The addition of 5 ml of water to give 90 per cent acetonitrile followed by washing with 10 ml petroleum ether was found to remove all but a trace of the fat without loss of DMDCS. Extraction from a 75 ml volume of petroleum ether solution was decided upon because if the volume were much less than 75, separation of the phases became quite slow; with a 75 ml volume there was no emulsion trouble and the phases separated rapidly.

When acetonitrile containing DMDCS was evaporated on a steam bath to dryness, or evaporated in the presence of small amounts of water or KOH solution, none or only traces of the DMDCS could be recovered. However, in an experiment where an approximately equal volume of water, 35 ml, and 0.5 ml of the KOH reagent were added to 45 ml of acetonitrile containing 1 mg of DMDCS and the resulting solution concentrated

on a steam bath under a current of air to a volume of ca 4 ml, about 88 per cent of the DMDCS was recovered. Evaporation of the acetonitrile in the presence of KOH solution at room temperature under a current of air gave variable results ranging from 50–85 per cent. The best results were obtained by removal of the acetonitrile *in vacuo* in the presence of KOH solution at temperatures below 55°C. However, the recoveries of added DMDCS were somewhat low, even from petroleum ether solution as shown in Table 4. At the 1 mg level the recovery was a little better than at the 0.5 mg level. It was thought that the low results might be due entirely to volatility, but the following experiment indicates that the degradation is inhibited slightly by the residual material in the KOH solution after removal of the acetonitrile: 45 ml acetonitrile, 5 ml H_2O, and 2 ml KOH reagent were placed in a round bottom flask, the flask immersed in a water bath at 35°C., and the acetonitrile removed *in vacuo* (water pump). The residual KOH solution was transferred to a 10 ml reaction cylinder and 400 micrograms of DMDCS added directly to the cylinder. The recovery of the DMDCS was only 90 per cent. When the experiment was repeated using freshly distilled acetonitrile, the recovery remained the same.

TABLE 4.—*Recovery of DMDCS added to 75 ml petroleum ether*

ADDED	FOUND	RECOVERY
mmg	*mmg*	*per cent*
250	200	80
	210	84
500	400	80
	430	86
1000	855	86
	890	89

The recovery of DMDCS added to acetonitrile was not improved by refluxing under a condenser with triethylamine or various amounts of KOH reagent as a fixative prior to removal of the acetonitrile *in vacuo*; generally, the results were poorer. Shaking the flask containing the acetonitrile and the KOH reagent, either hot or cold, prior to removal of the acetonitrile, failed to give results different from those obtained when the flask was not shaken.

The recovery of dimethyl chlorofumarate and dimethyl chloromaleate added to acetonitrile was determined. Two micromols of each (2 ml of aqueous solution) was added to different 45 ml portions of acetonitrile containing 3 ml water and 2 ml KOH reagent, and the acetonitrile removed *in vacuo* at 35°C. With the fumarate, 96 per cent recovery was obtained, but with the maleate only 75 per cent recovery was obtained.

TABLE 5.—*Recovery of DMDCS added to cheese; results corrected for the cheese blanks shown*

PRODUCT	ADDED	FOUND	RECOVERY
	p.p.m.	*p.p.m.*	*per cent*
Processed American	0	0.4	—
Processed American	1	0.7	70
Processed American	2	1.5	75
		1.6	80
Processed American	5	3.5	70
		4.0	80
Processed American	10	8.0	80
		8.1	81
Processed American	20	16.0	80
		16.4	82
Cheddar	0	0.4	—
	10	7.8	78
Swiss	0	0.3	—
	10	7.9	79
Processed Pimento	0	0.4	—
	10	9.2	92
Limburger	0	0.2	—
	10	7.8	78
Cream	0	0.2	—
	10	8.4	84

Typical recoveries of DMDCS added to various types of cheese at levels of 1–20 p.p.m. are shown in Table 5. The DMDCS was added in petroleum ether solution to the first 75 ml of extractant before it was blended with the cheese. All recoveries are corrected for the cheese blanks shown. The blanks on a number of other cheeses have been determined. They ranged from 0.2 to 0.5 p.p.m. apparent DMDCS when measured against the reagent blank.

Addition of 500 mg of Lloyd's reagent* to 10 ml solution failed to reduce the blank of 0.5 p.p.m. associated with one cheddar cheese sample. A trace of color in the case of cheese samples was sometimes present in the filtrate from the 10 ml degradation reaction solution. No such color

* Hydrated aluminum silicate adsorbent.

was found in the standard. This color did not contribute materially to the blank.

Dehydroacetic acid, which has also been proposed as an antimycotic for cheese and which contains a carbonyl group, was tested by the method. Ten mg added directly to the reaction cylinder and run through the method gave no color.

Application of the Method to Cheese Wrappers.—Recoveries of DMDCS added to single cheese wrappers (size necessary to wrap a half pound of sliced cheese) were quite satisfactory as shown in Table 6. Omission of the acetonitrile extraction step was tried, but with the type of wrapper used the recoveries were very poor, 50–60 per cent.

TABLE 6.—*Recovery of DMDCS added to waxed cheese wrapper*

ADDED	FOUND	RECOVERY
mg	*mg*	*per cent*
0	0	—
1	0.89	89
	0.90	90
3	2.67	89
	2.70	90

SUMMARY

A colorimetric method for the determination of dimethyl dichlorosuccinate and its possible degradation products in cheese is described. The method is based on the degradation of these compounds by KOH to pyruvic acid, which is then measured as the colored 2,4-dinitrophenylhydrazone in alkali. The test is sensitive to 1 p.p.m. of dimethyl dichlorosuccinate, and cheese samples known not to contain this substance gave no appreciable blank. Recoveries of DMDCS added to cheese at levels of 1 to 20 p.p.m. ranged from 70–92 per cent.

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STUDIES IN COAL-TAR COLORS—XIV: D&C RED NO. 39

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Among the colors listed as certifiable in the Coal-Tar Color Regulations (1) is a 2-[4-(β,β'dihydroxydiethylamino)-phenylazo]benzoic acid (Alba Red). This color is certifiable as D&C Red No. 39. The present paper describes the preparation of a purified sample of D&C Red No. 39. The purified sample has been used as a standard to investigate the validity of the titanium trichloride titration procedure for the quantitative determination of the pure color (2), and to determine the spectrophotometric properties of solutions of the dye. A spectrophotometric method for the simultaneous determination of the uncombined intermediates, anthranilic acid, and N,N'-(β,β'-dihydroxydiethyl)-aniline in batches of D&C Red No. 39 is also described.

EXPERIMENTAL

PURIFICATION OF INTERMEDIATES

Anthranilic acid.—Technical grade anthranilic acid was recrystallized twice from water, decolorizing with carbon. The final product was dried at 80°C. for 24 hours; m.p. 146.5–147.0°C. (lit. 147°C.) (3).

N,N'(β,β'dihydroxydiethyl)-aniline.—Technical grade N,N'(β,β'dihydroxydiethyl)-aniline was recrystallized three times from toluene. The final white crystalline powder was dried at room temperature under reduced pressure (2 mm Hg). The melting point of the purified product was 55.0–55.5°C.

PREPARATION OF D&C RED NO. 39

Purified anthranilic acid, 13.7 g (0.1 mole) was agitated with 30 ml of hot water, and then 25 ml of conc. hydrochloric acid was added. The soln was allowed to cool to room temp. Sufficient ice was added to lower the temp. to 0°C. and leave a small excess of ice. A soln of 7.0 g of sodium nitrite in 35 ml of water was added rapidly with stirring. After several minutes, the excess nitrous acid was destroyed with sulfamic acid. A soln contg 20.0 g (0.11 mole) of N,N'(β,β' dihydroxydiethyl)-aniline in 150 ml of 0.1 *N* HCl was prepared and cooled to 5°C. by adding ice.

The soln of diazotized anthranilic acid was added slowly, with continuous mechanical stirring, to the cold soln of N,N'(β,β' dihydroxydiethyl)-aniline. The soln was stirred at about 5°C. for one hour and was then allowed to warm to room temp., re-cooled to about 5°C., and slowly neutralized to *p*H 4.4–4.6 with dilute sodium hydroxide soln. The pptd color was recovered by filtration and was recrystallized three times from acetone. The product was dried at 80°C. for 24 hours. The melting point of the final product was 151.0–151.7°C.

ANALYTICAL DATA

Volatile matter (2 mm Hg. 30°C.) = 4.28%.

Nitrogen (semi-micro Kjeldahl): *Found* (moisture-free basis), 12.72%. *Calcd.* for $C_{17}H_{19}O_4N_3$: 12.75%.

Titration with $TiCl_3$: The A.O.A.C. procedure (2) gave a colorless end point.

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Ml of 0.1 *N* $TiCl_3$ required per g of dye: *Found* (moisture-free basis), 121.0, 121.2; *Calcd.*, 121.4.

SPECTROPHOTOMETRIC DATA

Spectrophotometric measurements were made with a Cary recording spectrophotometer. Weighed samples of about 0.1 g were dissolved in 25 ml of alcohol and diluted to 100 ml with water. Appropriate dilutions were made from these stock solutions.

Figure 1 shows the effect of *p*H on the absorbancy curve of the dye. The absorbancies in 0.05 *N*, 0.1 *N*, and 0.2 *N* hydrochloric acid solutions are identical. As the *p*H of the solution is varied from *p*H 2 to *p*H 7 there is a marked change in absorbancy curves. The absorbancy curves for solutions of *p*H 7, 8, and 9 are identical. The presence of two isosbestic points at

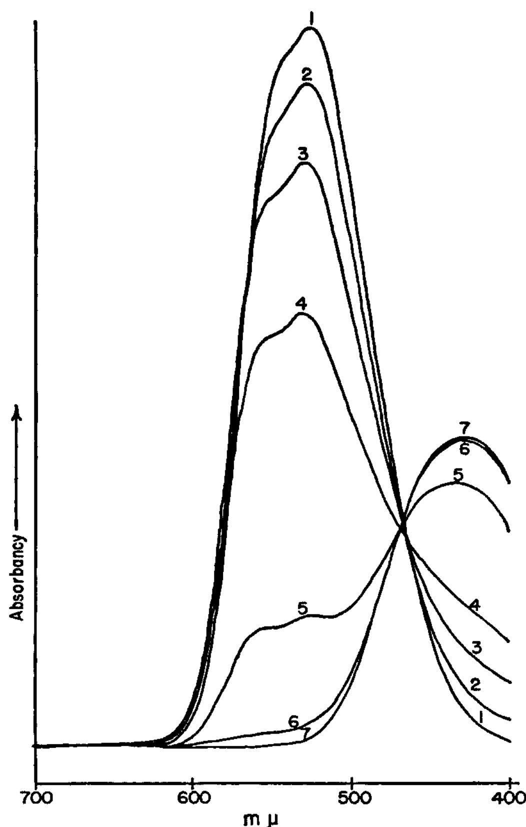

FIG. 1.—Absorbancy curves of D&C Red No. 39. Concn.: 10.50 mg./liter. Curve 1—0.1 *N* HCl; Curve 2—*p*H 2; Curve 3—*p*H 3; Curve 4—*p*H 4; Curve 5—*p*H 5; Curve 6—*p*H 6; Curve 7—*p*H 7 and 9.

approximately 466 and 470 mμ and the change in shape of the absorbancy curve with *p*H in the neighborhood of 540 to 560 mμ indicate the presence of three absorbing forms of the color in aqueous solutions.

Solutions of the dye in 0.1 *N* hydrochloric acid follow Beer's law to within ±0.9 per cent. In 0.1 *N* hydrochloric acid solution the average absorbancy of the color at the wave length of maximum absorbancy, 526–529 mμ, was 0.172 per milligram per liter. Solutions of the dye in 0.1 *N* hydrochloric acid are stable for at least 24 hours.

Several samples of commercial dyes were analyzed both by titration with titanium trichloride and by the spectrophotometric procedure, using the absorbancy data obtained from solutions of the purified sample. The percentages of pure dye obtained by titration were: 99.5, 98.8, and 96.0; the respective spectrophotometric results were 99.1, 97.9, and 95.8.

THE DETERMINATION OF UNCOMBINED ANTHRANILIC ACID AND N,N′(β,β′DIHYDROXYDIETHYL)-ANILINE IN D&C RED NO. 39

The uncombined N,N′(β,β′dihydroxydiethyl)-aniline in certifiable batches of D&C Red No. 39 must not exceed 0.2 per cent (1). There is no specific limit for the amount of uncombined anthranilic acid that may be present in a certified batch of the color; however, the presence of more than a fraction of one per cent of this intermediate in a sample of this color would cause the batch to be rejected as containing excess extractable material.

These intermediates are much more soluble in petroleum benzin than is the color. Extraction with this solvent provides a satisfactory procedure for separating the two intermediates from the dye.

Since there is a marked difference in the ultraviolet absorbancy curves of the two intermediates, simultaneous determinations of the two in a single solution is relatively simple.

METHOD

APPARATUS

Soxhlet extraction apparatus.

A spectrophotometer capable of isolating a 5 mμ wave length band suitable for measurements at 254 and 320 mμ.

REAGENT

Standard anthranilic acid solution (10 mg. per liter in 1% ammonia).

PROCEDURE

Place 500 mg of dye in a cellulose extraction thimble and ext. in the Soxhlet extractor with petroleum benzin for four hours. Transfer the ext. to a 500 ml separatory funnel, wash the extn flask with two 10 ml portions of petroleum benzin, and add the washings to the main ext. Ext. the combined ext. and washings with three 20 ml portions of 0.1 *N* hydrochloric acid. Not more than a trace of color is present in the aqueous soln at this point. Heat the combined aqueous extracts on a steam batch for 15 to 20 min. to remove any traces of the petroleum benzin, cool, add 2 ml

of concd. ammonia, and dilute to exactly 100 ml. Det. the absorbancy of the standard and unknown solns at 254 and 320 mμ with a suitable spectrophotometer.

CALCULATIONS

Calculate the amounts of the intermediates contained in the unknown from the equations:

$$aX + bY = As_{254}$$
$$cX + dY = As_{320}$$

where:

X = The concentration of anthranilic acid.
Y = The concentration of N,N′(β,β′dihydroxydiethyl)-aniline.
a = The absorbtivity of anthranilic acid at 254 mμ.
b = The absorbtivity of N,N′(β,β′dihydroxydiethyl)-aniline at 254 mμ.
c = The absorbtivity of anthranilic acid at 320 mμ.
d = The absorbtivity of N,N′(β,β′dihydroxydiethyl)-aniline at 320 mμ.
As_{254} = The absorbancy of the unknown at 254 mμ.
As_{320} = The absorbancy of the unknown at 320 mμ.

RECOVERY OF INTERMEDIATES

Purified samples of the intermediates, prepared according to the previous section, were used as standards. Figure 2 shows their absorption

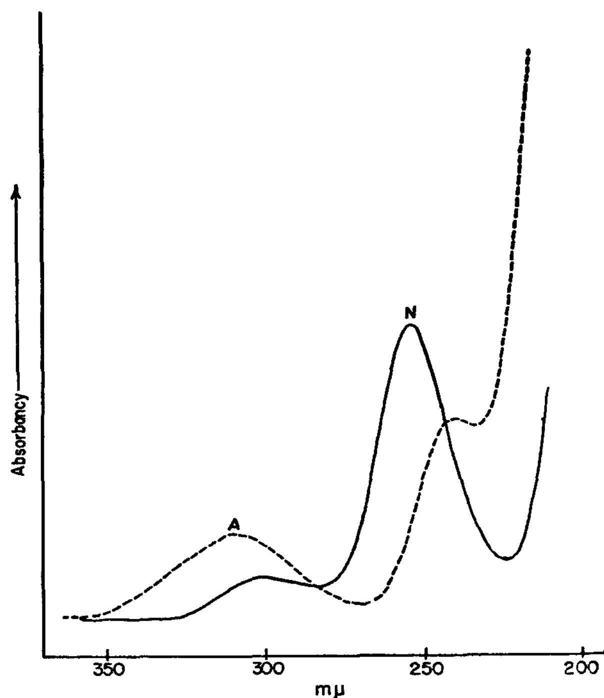

FIG. 2.—Absorbancy curves of anthranilic acid (A) and N,N′(β,β′-dihydroxydiethyl)-aniline (N) in 1 per cent ammonia solution. Concn. = 10.0 mg./liter.

TABLE 1.—*Recovery of anthranilic acid and N,N'(β,β'dihydroxydiethyl)-aniline*

ADDED		FOUND		NET		RECOVERY	
ANTHRANILIC ACID	N,N'(β,β' DIHYDROXYDIETHYL)-ANILINE	ANTHRANILIC ACID	N,N'(β,β'-DIHYDROXYDIETHYL)-ANILINE	ANTHRANILIC ACID	N,N'(β,β' DIHYDROXYDIETHYL)-ANILINE	ANTHRANILIC ACID	N,N'(β,β' DIHYDROXYDIETHYL)-ANILINE
mg	*mg*	*mg*	*mg*	*mg*	*mg*	*per cent*	*per cent*
—	—	0.12	0.43	—	—	—	—
—	—	0.10	0.40	—	—	—	—
0.5	2.0	0.56	2.31	0.45	1.89	90.0	94.5
0.5	2.0	0.54	2.22	0.43	1.80	86.0	90.0
0.5	2.0	0.56	2.50	0.45	2.08	90.0	104.0
1.0	1.0	1.09	1.31	0.98	0.89	98.0	89.0
1.0	1.0	1.05	1.60	0.94	1.18	94.0	118.0*
1.0	1.0	1.10	1.28	0.99	0.86	99.0	86.0
2.0	0.5	2.07	0.87	1.96	0.45	98.0	90.0
2.0	0.5	1.99	0.91	1.88	0.49	94.0	98.0
2.0	0.5	2.13	0.88	2.02	0.46	101.0	92.0
						Av. 94.0	Av. 94.0

* This result was disregarded in the average.

curves in 1 per cent ammonia solution as determined on a Cary Model 11 spectrophotometer. The concentration of each compound is 10.0 mg per liter of solution.

Known amounts of the intermediates were added to 500 mg portions of a composite sample of commercial D&C Red No. 39; the resulting mixtures were then analyzed by the proposed procedure. The recoveries are listed in Table 1. The average recovery of anthranilic acid was 94.4 per cent and that of N,N'(β,β'dihydroxydiethyl)-aniline was 94.2 per cent. The amount of color "bleeding through" into the final solution was negligible.

SUMMARY

A purified sample of D&C Red No. 39 has been prepared. Both the titanium trichloride titration procedure and the spectrophotometric method are satisfactory for the quantitative determination of the dye. Spectrophotometric data for aqueous solutions of D&C Red No. 39 have been presented. Aqueous solutions of the color in 0.1 *N* hydrochloric acid obey Beer's law.

A spectrophotometric method for the simultaneous determination of uncombined anthranilic acid and N,N'(β,β'dihydroxydiethyl)-aniline has been presented. The average recovery of added intermediates was 94 per cent.

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THE SEPARATION AND IDENTIFICATION OF CHLORINATED ORGANIC PESTICIDES BY PAPER CHROMATOGRAPHY

II. ALDRIN AND DIELDRIN

By Lloyd C. Mitchell and Wilbur I. Patterson (Division of Food, Food and Drug Administration, Federal Security Agency, Washington 25, D.C.)

This paper presents a method for the separation and identification of the insecticides aldrin and dieldrin by paper chromatography. Separation of the isomers of benzene hexachloride by a similar technique has recently been described (1).

METHOD

APPARATUS

(a) *Glass jar.*—For 8×8 inch sheets of Whatman No. 1 paper, accessories, drying rack, sprayer, etc. (the same as previously described (1)).

(b) *Filter paper.*—Whatman No. 1 in 8×8 inch sheets, washed with distilled water until halogen free, then air dried.

(c) *Brass funnel.*—With perforated plate for washing up to a ream of 8×8 inch paper sheets at a time.

REAGENTS

(a) *Stationary solvent.*—Refined soybean oil (free from fatty acids) in ethyl ether, A.C.S. grade (1+99, v/v).

(b) *Mobile solvents.*—Acetone, acetonitrile, ethanol, glacial acetic acid, methanol, methyl cellosolve, pyridine, or various combinations of these, with 20 to 30% water.

(c) *Development reagents.*—(1) 0.05 *N* silver nitrate in ethanol; (2) formaldehyde solution (ca 37%); (3) *N* potassium hydroxide in methanol; and (4) concentrated nitric acid with 30% hydrogen peroxide (1+1).

(d) *Standards**—Dissolve 36 and 365 mg of aldrin, and 38 and 381 mg of dieldrin, in 10 ml portions of ethyl acetate (for 0.01 and 0.1 molar solns of each). Prepare mixtures as needed. Keep in glass-stoppered bottles.

PROCEDURE

Spot the paper and develop the chromatograms, as described previously for benzene hexachloride, using 8×8 inch sheets (1). When the mobile solvent approaches (but does not reach) the top of the sheet (ca 1 to 4 hours, depending upon composition of the mobile solvent), remove paper from container, mark solvent front and hang sheet on rack in hood until dry (ca ½ hour for most solvents). Transfer dry paper to auxiliary glass rod in hood for spraying. Wearing rubber gloves, spray paper with the various developing reagents in the following sequence: reagent **(c)**(1) and air dry for ½ hour; reagent **(c)**(2) and air dry for ½ hour; reagent **(c)**(3) and transfer

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* Purified samples of aldrin and dieldrin were supplied by Julius Hyman Company Division, Shell Chemical Company.

TABLE 1.—*R_F values for aldrin and dieldrin obtained with various mobile solvents*

NUMBER	TIME OF RUN (HOURS)	MOBILE SOLVENT PHASE*	R_F VALUES							
			0.01 MOLAR CONCENTRATION				0.1 MOLAR CONCENTRATION			
			ALDRIN	DIELDRIN	ALDRIN AND DIELDRIN		ALDRIN	DIELDRIN	ALDRIN AND DIELDRIN	
1	1½	Acetone+Water 4+1	.34	.43			.32 .43	.44		
2	2¼		.39	.53			.32 .49	.50		
16	2¾		.48	.61	.49	.61	.47	.59	.46	.58
16	2¾		.48	.60	.49	.61	.46	.58	.45	.57
3	1	Acetonitrile+Water 7+3	—	.65			.30 .62	.62		
17	1		.40	.68	.44	.64	.35	.65	.38	.65
17	1		.38	.68	.42	.66	.32	.66	.38	.69
4	3½	Ethanol+Water 4+1	.30	.46			.26 .43	.43		
18	3½		.53	.68	.51	.68	.45	.63	.46	.65
18	3½		.53	.67	.48	.68	.49	.65	.50	.67
5	3¼	Glacial Acetic Acid+Water 3+1	.35	.57			.28 .56	.57		
19	3¼		.34	.58	.35	.60	.28	.56	.28	.53
19	3¼		.30	.57	.32	.57	.29	.55	.25	.50
6	3½	Methyl Cellosolve+Water 4+1	.20	.29	.20	.30	.19 .30	.24	.17	.27
7	3¼	Pyridine+Water 7+3	.48	.70			.41 .65	.67		
20	3¼		.43	.65	.44	.67	.39	.64	.39	.62
20	3¼		.46	.64	.46	.66	.42	.64	.41	.62
8	2½	Acetone+Ethanol+Water 3+3+2	.33	.48			.25 .47	.48		
21	3½		.43	.60	.44	.62	.38	.57	.36	.54
21	3½		.42	.64	.47	.65	.43	.68	.41	.58
9	2	Acetone+Methanol+Water 2+2+1	.28	.42			.25 .45	.43		
22	1¾		.29	.43	.29	.47	.29	.43	.27	.42
22	1¾		.30	.49	.31	.50	.29	.46	.27	.43

* Immobile solvent phase: Soybean oil+ethyl ether 1+99.

TABLE 1—(*continued*)

NUMBER	TIME OF RUN (HOURS)	MOBILE SOLVENT PHASE*	RF VALUES							
			0.01 MOLAR CONCENTRATION				0.1 MOLAR CONCENTRATION			
			ALDRIN	DIELDRIN	ALDRIN AND DIELDRIN		ALDRIN	DIELDRIN	ALDRIN AND DIELDRIN	
10	3¼	Acetone+Pyridine+Water 14+1+5	.36	.50			.28 .47	.47		
23	2		.36	.52	.36	.53	.31	.50	.32	.47
23	2		.34	.53	.37	.53	.36	.52	.34	.49
11	1½	Acetonitrile+Methanol+Water 2+2+1	.30	.48			.26 .46	.43		
24	1¼		.31	.47	.30	.45	.28	.44	.26	.43
24	1¼		.29	.48	.32	.50	.28	.48	.28	.46
12	2	Acetonitrile+Methyl Cellosolve+Water 2+2+1	.36	.54			.21 .51	.51		
25	1¾		.39	.59	.37	.56	.33	.55	.33	.53
25	1¾		.39	.59	.42	.60	.36	.57	.36	.56
13	2¼	Methanol+Pyridine+Water 15+1+4	.22	.38			.17 .34	.32		
26	2		.25	.39	.24	.43	.19	.38	.19	.35
26	2		.23	.42	.24	.43	.19	.38	.19	.35
14	1½	Acetone+Acetonitrile+Methanol+Water 1+1+1+1	.23	.39			.16 .36	.35		
27	1¼		.22	.38	.25	.38	.23	.38	.21	.36
27	1¼		.23	.40	.25	.40	.23	.39	.23	.38
15	3	Acetone+Ethanol+Methanol+Water 1+1+1+1	.23	.38			.20 .36	.32		
28	3		.29	.45	.29	.47	.26	.42	.22	.37
28	3		.27	.49	.33	.52	.30	.46	.24	.44
29	2½		.25	.40	.24	.41	.22	.38	.21	.35
29	2½		.27	.45	.28	.45	.23	.40	.22	.37

* Immobile solvent phase: Soybean oil+ethyl ether 1+99.

paper on glass rod to rack, heat immediately in oven at 130–133°C for ½ hour, cool, return sheet to auxiliary rod in hood, spray with reagent (c)(4) and air dry over night. Finally, expose paper to the sun.

RESULTS AND DISCUSSION

Detection of spots.—The indicator used for benzene hexachlorides failed to show any reaction on the chromatograms of aldrin and dieldrin. (Increasing the concentration of the alkali and heating at higher temperatures for longer periods of time failed to develop spots of silver chloride.) Treatment with other chemicals which might be expected to serve as dehalogenating agents (nitric acid, chromic acid, potassium dichromate, hydrogen peroxide), separately or in various combinations, also failed to split off chlorine. However, when formaldehyde solution was introduced into the sequence of sprays, spots appeared for both aldrin and dieldrin (2). The spots were darker and more intense when drying took place at temperatures of 35–37°C. rather than at 23–25°C. Some variations in the recommended procedure are possible: the silver nitrate can be dissolved in the formaldehyde solution if used fresh; it should be on the paper prior to formaldehyde treatment, or sprayed simultaneously with it. Heavier deposits of silver chloride seem to be obtained when the paper is sprayed with the potassium hydroxide solution while still "damp" with formaldehyde. The purpose of the hydrogen peroxide is to bleach the yellow color imparted to the paper by potassium hydroxide treatment at 130–133°C.

In an effort to find out why formaldehyde was an essential reagent in the development of the spots, other potential reducing groups—formate, hydrazine, hypophosphite, and sulfite—were tried, with uniform failure to show any spots.

Solvent systems.—The solvent system [acetic anhydride (immobile) and isooctanes (mobile)], used to separate the isomers of benzenehexachloride (1), was found unsatisfactory at the time this study was undertaken because the mobile solvent could not be used at the prevailing temperature (ca 35°C.). Later, when room temperature was about 23°C., this solvent system was found to separate aldrin and dieldrin with R_F values of about 0.95 and 0.84, respectively. Combinations of vegetable, animal, or mineral oils, as immobile solvent, and such water miscible solvents as acetone, acetonitrile, ethanol, glacial acetic acid, methanol, methyl cellosolve, pyridine, or various mixtures thereof, with twenty to thirty per cent water, as mobile solvent, also afford a separation of aldrin and dieldrin. Without water as a component, aldrin and dieldrin travel with or near the solvent front, and the immobile solvent likewise ascends the paper in varying degree depending upon its solubility. The R_F values of aldrin and dieldrin decrease with increasing amounts of water in the mobile solvent. Too much water in the mobile solvent or excessive concen-

tration of the test material on the paper causes streaking.

Soy bean oil was selected as the immobile solvent in preference to tung oil or linseed oil, which interfere with absorption of the indicator reagents. Soybean oil gave somewhat more intense spots than peanut oil or mineral oil. Palm oil, solid at room temperature, gave an erratic solvent front and R_F values. Corn or cottonseed oils were not tried.

The optimum concentration of immobile solvent on the paper lies between somewhat narrow limits. If too much is added, the indicator reagents will not "wet" the paper; if too little, separation of aldrin and dieldrin is unsatisfactory. The spraying procedure (1) is preferred for control of the concentration of immobile solvent on the paper rather than other procedures which require removal of excessive amounts first added (3, 4, 5). The optimum amount of immobile solvent on the paper is approximately 2 mg per square inch.

A number of experiments were made, varying the volume of water in the mobile solvents from twenty to eighty per cent in steps of ten or twenty. As the proportion of water increased, the R_F values decreased; streaking became increasingly prominent and in some instances there was no movement of the aldrin and dieldrin. Experiments which indicated optimum separation, with R_F values within the middle third or middle half of the paper, were repeated. Earlier experiments (number 1 to 15) were made at room temperatures of 35°C., or above; the later ones (16 to 29) at 25° or less. With some mobile solvent mixtures this variation in temperature produced material differences in R_F values, while with other solvents little or no difference resulted. The higher temperatures gave better results.

Table 1 shows the mobile solvent systems (in groups of two, three, or four components, including water) which were more or less satisfactory. The 0.01 and 0.1 molar concentrations of aldrin and dieldrin, and mixtures thereof, were run on two different papers in experiments numbered 1 to 15; both papers, however, were run simultaneously. Acetone+water (4+1) or acetonitrile+water (7+3) gave the most compact spots and are the recommended mobile solvents. As is indicated in the table in the column for 0.1 molar aldrin, chromatograms run at about 35° show two spots; the one with lower R_F value is much larger than the second. The R_F value for the second spot invariably coincided with that of dieldrin.

Spots of 0.1 molar solution did not give as compact spots or as complete separation as the 0.01 molar solutions; this is presumably the result of "overloading" the paper.

(Various mobile solvents besides those listed in the table were tried; these were unsatisfactory because they either dislodged the immobile solvent or gave poor separation. Included among these were methyl, *n*-propyl, iso-propyl and *n*-butyl alcohols, diisopropyl ketone, methyl propyl ketone, ethyl carbonate, and collidine.)

SUMMARY

Mixtures of aldrin and dieldrin are separated by an application of paper chromatography in which: (1) the paper is pre-impregnated by spraying it with an ethyl ether solution of the stationary phase (soybean oil), and (2) any one of a number of water miscible organic solvents is employed as the mobile phase. Acetone plus water (4+1) or acetonitrile plus water (7+3) are the preferred mobile solvents. These substances are detected on the paper by dechlorination in the presence of silver nitrate. The dechlorination takes place when the paper is sprayed in sequence with (a) silver nitrate, (b) formaldehyde, and (c) potassium hydroxide. Discoloration of the paper is removed by spraying it with a mixture of concentrated nitric acid and 30 per cent hydrogen peroxide. The paper is finally exposed to the sunlight. As little as 20 micrograms of each substance may be clearly separated.

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NOTE

NOTE ON THE DETERMINATION OF ASH IN NON-FAT DRY MILK SOLIDS*

By ROBERT E. CANNY (Food and Drug Administration, Federal Security Agency, Minneapolis 1, Minnesota)

Two methods for the determination of ash in non-fat dry milk solids are available in *Official Methods of Analysis*, Seventh Edition, 1950. In the first method (**15.96**), one gram of sample is ashed at 550° until free from carbon, while in the second (**15.97**), two grams of sample are ashed for one hour at 550°C., wetted down with water, broken up, dried on the steam bath, and re-ashed for one hour at 550°. The former method is used in studies on composition, or as the initial step in the determination of individual ash constituents, while the latter method is used prior to a determination of the alkalinity of the ash.

The question arose as to whether ash values obtained by method **15.97** were identical with those obtained by **15.96**. If this were the case, a single sample would suffice for the determination of both the ash and the alkalinity of the ash.

Eighteen samples of neutralized and unneutralized non-fat dry milk solids were analyzed in duplicate or triplicate by both methods. The results are given in the following table:

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* Presented at the annual meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., Sept. 29–30 and Oct. 1, 1952.

SAMPLE NUMBER	SUB. NUMBER	PER CENT ASH BY A.O.A.C. 15.96		PER CENT ASH BY A.O.A.C. 15.97	
		PER CENT ASH	AVERAGE	PER CENT ASH	AVERAGE
91-150 K	1	8.96 8.99 9.01	8.99	9.04 9.02 9.03	9.03
	2	9.04 8.96 9.01	9.00	9.02 9.02 9.02	9.02
	3	8.12 8.19 8.10	8.14	8.12 8.07 8.15	8.11
85-671 K	1	8.10 8.15 8.12	8.12	8.10 8.14 8.11	8.12
75-813 K	1	8.14 8.09 8.14	8.12	8.12 8.14 8.13	8.13
75-814 K	1	8.26 8.26 8.29	8.27	8.26 8.27 8.30	8.28
91-385 K	2	9.37 9.41 9.41	9.40	9.32 9.35 9.26	9.31
	3	8.95 9.00 8.96	8.97	9.02 8.99 8.91	8.97
34-686 L	1	8.80 8.82	8.81	8.81 8.81	8.81
	2	8.74 8.80	8.77	8.70 8.59	8.65
	3	8.98 8.95	8.97	9.04 8.89	8.97
48-378 L	1	7.90 7.93	7.92	7.90 7.94	7.92
	4	7.96 8.02	7.99	8.01 8.09	8.05
48-372 L	2	7.72 7.68	7.70	7.68 7.62	7.65
48-375 L	3	7.28 7.38	7.33	7.39 7.21	7.30
91-393 K	2	8.06 8.09	8.08	8.10 8.01	8.06
85-390 K	1	7.78 7.83	7.81	7.89 7.84	7.87
85-391 K	1	8.04 8.13	8.09	8.12 8.08	8.10

Statistical analysis of the data indicated that there was no significant difference between the results obtained by these two methods for the determination of ash in non-fat dry milk solids.

ACKNOWLEDGMENT

Grateful acknowledgment is made to Mr. Howard Edelson of the Statistical Branch of the Division of Food for performing the statistical analysis.

BOOK REVIEWS

Insects: The Yearbook of Agriculture, 1952. By ALFRED STEFFERUD, Ed., *et al.*, United States Department of Agriculture, Washington, D. C., 1952. 780 pp., illus., 72 col. pl., $2.50. (For sale by Sup't. of Documents, Wash., D. C.)

This book is the latest issue of a series that has a venerable and distinguished background. It is the 105th volume of an annual work on agriculture issued with varying titles by the United States Government over a period of 112 years, from 1840 to 1952, inclusive (with 1847, 1882, 1944–47 and 1951 representing 7 consolidations with other years). It will be remembered in this connection that our Department of Agriculture originated in 1839 as a section of the Patent Office, became a separate Department in 1862, and was raised to Cabinet rank in 1889. We find, therefore, that from 1840 to 1861, the series was designated as "Report of the Commissioner of Patents;" from 1862 to 1893 it was "Report of the Commissioner of Agriculture;" from 1894 to 1925 inclusive it was "Yearbook of the U. S. Department of Agriculture;" while from 1926 to 1952 inclusive it has simply been "Yearbook of Agriculture." During earlier years the books comprised a rather desultory collection of statistics, more or less technical essays, letters, and fiscal and miscellaneous information. After 1894, however, the purely business and administrative subject matter was issued in a separate volume known as the "Annual Report," and the "Yearbook," as such, thereafter comprised various abstracts and digests of technical research performed by the Department. This most recent volume entitled "Insects" takes its place in the "Yearbook" subject series that began in 1936, and which has dealt successively each year thereafter with plant and animal genetics, soils, nutrition, economics, climate, livestock diseases, developments in agricultural sciences, grass, trees, and the processing of farm products.

An excellent idea of the general scope of the new volume can be gained by a brief survey of its contents. In addition to the introductory matter, there are 21 general subdivisions, each containing from 4 to 11 papers prepared by specialists on the given subjects. These are followed by a section containing 72 page-size plates in full color of all the more important of the insects affecting American agriculture and public health. Each of these bears a brief discussion of the insect's life history, habits, and control measures, and the format is such that each can be issued later as a separate leaflet for popular use in schools and elsewhere. The various subdivisions of the book deal with such matters as "How to Know an Insect," and contain papers written by Muesebeck, Mickel, and Oman; "Insects as Helpers," by Bishopp, Vansell, Roberts, Todd, and others; "Insects as Destroyers," by Haeussler, Foster, Giltner, Christenson, and others; "The Nature of Insecticides," by Roark, Haller, Carter, Chapman, Sullivan, Fulton, and others; "Applying Insecticides," by Newcomer, Westlake, Landis, Messenger, Popham, Irons, and others; "Warnings as to Insecticides," by Bishopp, Horsfall, Boswell, Porter, Reed, Dunbar, and others; "Resistance to Insecticides," by Porter, Bruce, and King; "Fumigants," by Cotton, Lane, Latta, and Chisholm; "Quarantines," by Burns, Swain, Becker, Conkle, and Messenger; "Other Controls," by Clausen, Steinhaus, Burks, Richardson, Baker, Packard, Mathews, and others; "Economic Entomology," by Davis, Leiby, Searls, Jones, and others; "Insects, Man and Homes," by Henderson, Stage, and Knipling; "Insects on Cotton," by Rainwater, Gaines, Curl, White, and Ewing; "Insects and Vegetables," by Roberts, Brindley, Chamberlin, Cook, Douglass, and others; "Insects on Fruit," by Carter, Hoidale, Hadley, Fleming, Middleton, and others; "Insects on Field Crops," by Packard, Parker, Wakeland, Bradley, Caffrey, and others; "Pests on Ornamentals," by Weigel, St. George, and Smith; "Livestock and Insects," by Eddy, Knipling, Bruce, Laake, and Roberts; "Forests, Trees and

Pests," by Brown, Keen, May, and others; and "Insects and Wildlife," by Cope, Lindquist, Linduska, and Kalmbach.

Of special interest to the readers of this review are those papers dealing with insecticides and control, and these include discussion of such subjects as "How Insecticides are Developed," "How Insecticides are Mixed," "How Insecticides Poison Insects," "The Organic Insecticides," "The Inorganic Insecticides," "Insecticides from Plants," "Oil Sprays for Fruit Trees," "Aerosols and Insects," "Research on Aerial Spraying," "Machines for Applying Insecticides," "Choosing and Using Hand Equipment," "The Safe Use of Insecticides," "Toxicity to Livestock," "Residues, Soils and Plants," "Residues on Fruits and Vegetables," "State Pesticide Laws," "The Federal Act of 1947," "Insecticides and the Pure Food Law," "Insecticides and Flies," "Mosquitoes and DDT." "Nature and Use of Fumigants," "Fumigating Soils and Plants," and "Fumigating Stored Foodstuffs." In papers which deal with the newer insecticides, particular attention has been given to full discussion of the results of most recent investigations, especially those likely to be of greatest practical value to the farmer, the county agent, and other non-technical people. All of the more important articles are followed by a list of selected references for those who may desire to make further study of the subject, and there is also included in the volume a general bibliography on insects, which covers both economic and systematic works arranged under respective sub-heads. Of note-worthy value are the "Conversion Tables and Equivalents," "The Summary of Federal Plant Regulatory Legislation," and the latest approved list of names and symbols (including trade names) of the newer insecticides.

On the whole there has been brought together into this 1952 Yearbook on Insects the boiled-down results of approximately one hundred years of research by the Bureau of Entomology and Plant Quarantine and the co-operating State agencies which were responsible in large measure for the preparation of this book. The century has seen great changes in farming methods, the intensiveness and extent of agriculture, transportation, and crops; all of these have profoundly affected our relationships with insects. It is the purpose of this book to contribute to better understanding of these relationships and to the efficiency and well-being of American farming and living.

Biochemical Preparations, Volume II (1952). E. G. BALL, Editor. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, New York. vii+109 pp. Price $3.00.

This volume gives methods of preparation for twenty-three compounds (and several intermediates) of biochemical interest.

For example, the preparation of l-α-glycerophosphoric acid goes by this method: d-mannitol→1,2,5,6-diacetone+d-mannitol→d-acetone glyceraldehyde→d-acetone glycerol→l-α-glycerophosphoric acid.

The format is satisfactory and no typographical errors were noted. The book has a cumulative index and the reference list to compounds in *Organic Synthesis* will be convenient for many readers.

N. ETTELSTEIN

Chemical Control of Insects. By T. F. WEST, J. ELIOT HARDY, and J. H. FORD. John Wiley & Sons Inc., New York, 1953. 12 mo., cloth, 211 pp., illus., $3.25.

The originally issued first English edition of this little book came out some time ago, and has already been briefly noticed in *This Journal*, **35**, 807 (1952). Sufficient space, however, is here given to listing this newly issued first American edition so that its availability here in America may be known to our readers who on short

notice might have urgent need for this work of outstanding usefulness. Aside from a greatly improved and more attractive format, there appear to be no changes of importance from the previous English edition. The various methods using the newer insecticides are well described and their chemistry is detailed. Again the authors emphasize that the possibilities for use of still other new and useful synthetic compounds are far from being exhausted. The diversity of reactions to insecticides that continue to be found within the insect groups, and the generalizations the authors have made therefrom, make this book as stimulating as it is useful.

J. S. WADE

Maleic Anhydride Derivatives. By LAWRENCE H. FLETT and WILLIAM H. GARDNER. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, New York (1952). x+269 pp. Price $6.50.

The chemist faced with a problem of synthesis of an organic compound has in this book a compilation of 116 different reactions involving maleic anhydride or one of its simple unsaturated derivatives. The authors have indicated that this is not intended for a textbook nor a monograph; instead, it is intended to present a brief review of the various types of reactions afforded principally by the maleyl group, with a single example of each.

The reactions are divided among eight chapters headed according to the class of the reactant: Hydrocarbons, Halogens and Their Compounds, Hydrogen, Metallic Compounds, Compounds containing Nitrogen, Compounds containing Oxygen, Sulfur Compounds, and Energy and Catalysts. Each chapter commences with a two-page introduction to the activity of the particular reactant class with respect to the double bond of maleic anhydride, and with generalizations and brief explanations of the mechanisms of the specific reactions. Each reaction occupies two facing pages: the left-hand page deals with the product formed in the preparation and includes a discussion of the method of preparation, the uses of the product, and a description of several homologous compounds. The facing page is concerned with a brief description of the specific method of preparation and selected references written in a manner that has been made familiar by the volumes of *Organic Reactions*.

In the opinion of this writer the authors were successful in their effort to present a lucid and easily read résumé of the reactions involving the maleyl group. The format lends itself to a rapid perusal of a number of varied reactions. It would seem, however, that this book could be improved considerably by the inclusion of tables listing all the known compounds prepared by the various reactions discussed, with their references.

S. M. HESS

Organic Syntheses, Volume 32. RICHARD T. ARNOLD, Editor-in-Chief. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, N. Y. vi+119 pp. Price $3.50.

Volume 32 of Organic Syntheses contains 43 carefully tested and detailed synthetic procedures for a wide variety of organic compounds. Each procedure includes laboratory instructions, special precautions to be observed with hazardous materials, descriptions of any unusual apparatus employed, and references to the original literature. Expected yields are given for the crude products, and for each step in their purification. In addition, alternative methods of preparation are mentioned, and the extension of each synthesis to compounds of analogous structure is discussed.

The following compounds are included in volume 32:

Abietic Acid
Acrolein Acetal
Alloxan Monohydrate
2-Aminobenzophenone

ϵ-Aminocaproic Acid
1,1′-Azo-*bis*-l-cyclohexanenitrile
β-Bromoethylphthalimide
tert-Butyl Hypochlorite
1-Chloro-2,6-dinitrobenzene
p-Chlorophenyl Salicylate
β-Chlorovinyl Isoamyl Ketone
Cyanogen Iodide
3-Cyano-6-methyl-2(1)-pyridone
1,2-Eyclohexanedionedioxime
Cyclohexene Sulfide
Cyclopentadiene and 3-Chlorocyclopentene
Phenylacetamide
2,4-Diamino-6-hydroxypyrimidine
2,2-Dichloroethanol
1,1′-Dicyano-1,1′-bicyclohexyl
1,2-Di-1-(1-cyano)-cyclohexylhydrazine
Diethyl Δ^2-Cyclopentenylmalonate
Diethyl Ethylidenemalonate
Dimethyl Acetylenedicarboxylate
4,6-Dimethylcoumalin
5,5-Dimethyl-2-pyrrolidone
asym-Dimethylurea
α,α-Diphenylsuccinonitrile
2-Ethylhexanonitrile
Ethyl Orthocarbonate
Flavone
1,1′-Ethynylene-*bis*-cyclohexanol
Isodehydroacetic Acid and Ethyl Isodehydroacetate
β-Ketoisoöctaldehyde Dimethyl Acetal
Methyl *p*-Acetylbenzoate
Methylglyoxal-*w*-phenylhydrazone
Methyl α-Methyl-α-nitrovalerate
Naphthalene-1,5-disulfonyl Chloride
Neophyl Chloride
10-Undecynoic Acid
β-Tetralone
Sodium Nitromalonaldehyde Monohydrate
Thiobenzoic Acid

C. S. PRICKETT

Advances in Agronomy. Edited by A. G. NORMAN. University of Michigan, Ann Arbor. Prepared under the auspices of the American Society of Agronomy. Academic Press, Inc., New York, N. Y. (1952). 8 vo. cloth, 416 pp., illus. Price $8.50.

This volume, the fourth within its series, deals with specialized topics on recent progress in the theory and practice of field-crop production and soil management. It contains nine articles, of varying length and range, all fully referenced, and prepared by fourteen specialists in their respective fields. Some of the papers go beyond what is normally considered Agronomy, and some are more specialized than others, but it is recognized that the definition of what constitutes a specialized article depends greatly upon the personal background and interests of the reader. While all of these articles contain excellent material this notice is limited to the four articles of the series whose subject matter lies more nearly within the usual scope of interests of *This Journal*.

The first of these is entitled "Type of soil colloid and the mineral nutrition of plants," by A. Mehlich and N. T. Coleman, North Carolina Agricultural Experiment Station, Raleigh. In addition to the introductory matter, there is discussion of the ionic environment of root plants in the soil, including both ion exchange and ion activity. The growth and cation contents of plants grown in natural and synthetic soils are discussed regarding degree of base saturation, associated metal cations, cation exchange capacity, and the ecological array of plants. Agronomic applications are indicated, and it is pointed out that recognition of the type of colloid as one of the factors influencing the availability of ions and hence of crop yield and quality would be expected to have important bearing on lime and fertilizer practices.

The second of these articles is "Copper in nutrition," by F. A. Gilbert, Battelle Memorial Institute, Columbus Ohio. A historical survey of the subject emphasizes the recent rapidity of the advance of copper, along with cobalt and one or two of the other elements, into prominence in the field of nutrition—from supposed poison to nutrient in twenty-five years. Discussion includes the value of copper to the plant

and effects of copper deficiency in plants; the factors affecting amount, availability, and effects on crop yields of copper in the soil, as well as its residual and possible toxic effects and its effect on availability of other elements. The necessity of copper to animal life also is pointed out, and its use in the body, its toxicity, and its value as an anthelmintic and in mineral supplements are indicated. Attention likewise is given to the status of regions of copper deficiency over the world, particularly where poor crop growth appears to be due to mineral unbalance, rather than to actual deficiency of any one element. The various remedies to use, or methods to avoid, particularly regarding highly concentrated salts or fertilizer in bringing about desired changes are given.

The third of these articles is entitled "Soil manganese in relation to plant growth," by E. G. Mulder and F. C. Gerretsen, Agricultural Experiment Station and Institute for Soil Research, T. N. O., Groningen, The Netherlands. Among the several factors here considered are the various usual methods of manganese determination, the availability of soil manganese and its estimation by chemical analysis, the role of microorganisms in transforming manganese compounds, and the symptoms of and methods for correcting manganese deficiency in plants. Survey also is made of manganese nutrition and fertilizer interactions, and to manganese toxicity in plants, particularly the effect of nitrogen compounds, of phosphorus, of calcium, of manganese excess in relation to iron deficiency, and manganese toxicity in relation to molybdenum supply. Likewise discussed are manganese in relation to carbohydrate breakdown and to nitrogen metabolism, and the role of manganese in photosynthesis.

The fourth of these articles is entitled "Vegetation control on industrial lands," by K. C. Barrons, Dow Chemical Company, Midland, Michigan. This paper outlines the scope and nature of the problem as a whole, and the advances that have been made in the problems involved in dealing with industrial lands. However, more important is the rather detailed discussion therein of the chemicals recently used for vegetative control. These include chlorophenoxyacetic acids, sodium chlorate, sodium trichloroacetate, substituted phenols, herbicidal oils, boron compounds, sodium arsenite, ammonium sulfamate, and various mixtures of herbicides. It is emphasized that only by a working knowledge of plant taxonomy and ecology, in addition to the new technology related to herbicides, can the most efficient vegetation control be accomplished.

The remaining five articles are: "Grassland agronomy in Australia," by H. C. Trumble, "Physiological basis of variation in yield," by D. J. Watson, "Ecological and physiological factors in compounding forage seed mixtures," by R. E. Blaser and others, "Atomic energy and the plant sciences," by N. E. Tolbert and others, and "Soil and the growth of forests," by T. S. Coile.

In addition to the Editor, the Advisory Board in the preparation of this volume comprised J. E. Adams, I. J. Johnson, Randall Jones, C. E. Marshall, R. Q. Parks, K. S. Quisenberry, V. G. Sprague, and E. Winters. They have produced an excellent book. In making this series available to students and others, the American Society of Agronomy has rendered a valuable public service.

J. S. Wade

Detergents—What They Are and What They Do. By Donald Price. Chemical Publishing Co., Inc., 212 Fifth Avenue, New York, N. Y. (1952). vii+159 pp. $4.00.

This book contains a considerable amount of information about detergents and detergency. It will be of interest to the chemist who wishes to be generally informed on the why and wherefor of the various products on the market.

The information is presented in elementary technical form. The cover suggests that the book will be of value to the intelligent housewife. This reviewer doubts that the housewife without technical education would understand much of the contents.
G. ROBERT CLARK

Methods of Statistical Analysis, 2nd Edition. By CYRIL H. GOULDEN. John Wiley & Sons. 467 pp. $7.50.

This is the second edition of a work originally published in 1939. Comprehensive revision and inclusion of much new material have substantially altered its character, and it may be considered as essentially a new book.

The author has included familiar chapters on tests of significance, analysis of variance, regression analysis, correlation, covariance, goodness of fit, and chapters on basic and complex experimental design. In addition, there is an excellent chapter dealing with non-orthogonal data. This is an important but often sadly ignored subject of concern to the experimenter or the analyst who is confronted with data containing missing observations, or disproportionate numbers of observations, in the test groups. A chapter on quality control serves as an introduction to those who must seriously concern themselves with that aspect of statistics. An unusual newcomer to this type of text is the chapter dealing with a segment of biological assay technique, that of probit analysis.

In the preface the author has succinctly expressed his views concerning those for whom the book is primarily intended when he states that "the subject matter is slanted rather definitely toward the needs of the student who is now, or will eventually be, a research worker." We are inclined to agree with this viewpoint. The wide range of subject matter encompassed by the text is admirable from the point of view of the student, but for the research worker with specialized problems, the analogy of the rifle being perhaps a more potent weapon than the shotgun is brought to mind. Those concerned with quality control need far more detailed information than is given in the chapter on that subject. The experimenter dealing with biological problems very often uses other methods than the probit. This is not a criticism of the book but an attempt to point out its limits of interest.

The individual chapters are developed in a highly lucid manner, both verbally and algebraically. Perhaps the most outstanding feature of the book is the profuse use of completely-worked out examples—an invaluable aid in understanding the techniques. Since the author is primarily concerned with investigations in the field of agriculture, it is quite natural that the illustrations are agricultural in context.

The basic statistical tools are made available in this book. They are certainly of aid in developing statistical thinking and the statistical design and analysis of experiments.
WILLIAM WEISS

Soil Microbiology. By S. A. WAKSMAN. John Wiley & Sons, Inc., 440 Fourth Avenue, New York 16, N. Y., 1952. vii+356 p. $6.00.

This intermediate textbook presents in logical sequence the relationship of soil microorganisms to soil fertility. It is written in pleasing style and is well documented with data, many from the author's own investigations. Each chapter is provided with a list of key references, well up to date, which compensate for descriptions necessarily elementary in covering so vast a field in a limited number of pages. The beginnings of soil microbiology are traced from the contributions and controversies of the early chemists, physiologists, microbiologists, and agronomists, and through the Golden Age of brilliant contributions on specific soil microbial functions. Recent developments are also covered. Present information is considered sufficient to im-

part independence to the science and to establish its ecological, physiological, agronomical and pedological phases.

The soil as a culture medium is discussed in connection with the microbial population as a whole. Methods of study are briefly outlined. Specific soil microorganisms are reviewed under a physiological classification that emphasizes significant activities of various morphological types, including actinomycetes, higher fungi and protozoa, and the true bacteria. Organic decomposition in soil and composts is considered on the basis of the chemical nature of plant and animal residues. The importance of specific organisms and environmental factors, especially free oxygen supply, in determining the end-products is well illustrated by graphs and tables. A number of simplified type reactions are presented. Metabolic outlines more completely presented would have enhanced the value of this chapter, which serves also as an introduction for the following discussion of humus and its decomposition. Humus is considered as a natural organic system in dynamic equilibrium with the microorganisms and environment. It is emphasized that humus is not a final and stable end-product and is itself slowly decomposed. The resulting gradual release of plant nutrients is taken into account as an important function in soil fertility; the physical, chemical, and biological effects are also noted. The abundance and nature of humus in different soils is briefly discussed. Peat and forest humus and the clay-humus complex are little more than mentioned. While Dr. Waksman's "Humus" is listed in the key references, it would have been appropriate for him as an outstanding authority to have presented a more elaborate treatment here. The chapter on decomposition of soil organic matter and evolution of carbon dioxide is fundamental. Graphs and tables are well chosen to illustrate how rate and extent of CO_2 evolution correlate with decomposition under various conditions. Qualitative as well as quantitative aspects are considered. A later chapter on manures and composts supplements the chapters on humus and organic decomposition.

Nitrogen transformations in the soil are outlined under protein decomposition, ammonification, nitrification, denitrification, and nitrogen fixation. The biology, biochemistry, and agricultural importance of symbiotic and nonsymbiotic fixations are well presented and are supported by pertinent data and illustrations. The section on microbial transformation of minerals deals chiefly with sulfur oxidation, liberation of phosphate from organic combination, and solvent action on insoluble phosphates. Arsenic, selenium, silicon, and the trace elements are mentioned in passing.

Various interrelationships between soil microorganisms and higher plants are dealt with in a chapter emphasizing complexity of the soil population. Importance of the rhizosphere, or root zone of increased microbial activity, and of influences resulting from CO_2 production are discussed. Associative and antagonistic effects are shown to lead to mutual equilibrium in the microbial complex. Production of antibiotics is covered briefly but authoritatively. A comprehensive chapter on disease-producing microorganisms in the soil deals extensively with plant pathogens and their control. The final chapters consider the relationships of microorganisms to various aspects of soil fertility and conservation. In conclusion, it is reemphasized that the soil is a highly complex, living system and that soil microbiology is a broad borderline science.

Few faults are to be found. The reactions presented on page 185 to illustrate denitrification are misleading. Nitrate, and in some cases nitrite, may serve as a hydrogen acceptor for facultative bacteria in the absence of free oxygen, but O_2 is not liberated. The use of Fig. 89, representing an alleged life cycle of the nodule bacteria, is unfortunate. While these symbiotic nitrogen fixers exhibit morphological

and cytological changes that constitute a growth cycle, the bacteroids have been shown to be incapable of reproduction; a cyclogenic life history does not occur. Exception may be taken to the statement, on p. 165, that were microorganisms "less active in the liberation of CO_2 from the dead plants and animals—the limited (atmospheric) supply of CO_2 having been exhausted would gradually lead to an end of all forms of life." Microorganisms may act "as regulators of CO_2 in the atmosphere and of the amount available to plants" under limited conditions but the effect would be localized, generally to a thin layer immediately above the soil. Geochemists have established that volcanic action supplies more than 90 per cent of the atmospheric CO_2 in juvenile state, and that the remarkably constant concentration is maintained by the calcium carbonate-bicarbonate buffer system in the seas. This also emphasizes the mineral nature of CO_2 and the biologically independent nature of autotrophic organisms, which can use this mineral as a sole source of carbon. Biologists could well avoid use of the misnomer, "inorganic carbon."

The author's objective of presenting "a broad outline . . . , a philosophy of soil microbiology" is well achieved. The book will appeal to the specialized student as well as to the general reader seeking information on the biodynamics of the soil.

W. B. BOLLEN

Ice Creams and Other Frozen Desserts. By J. H. FRANDSEN and D. HORACE NELSON. J. H. Frandsen, Amherst, Mass. 282 pp. Price $5.50.

This book is written for the ice cream trade and thereby serves as a simple and readable introduction to manufacturing and trade practices. It discusses definitions, composition, ingredients, flavorings, calculations, manufacturing, packaging, and distribution. There is a chapter on sanitation, with emphasis upon plant and equipment, but little attention is paid to the cleanliness of the cream. The sediment test is covered by a photograph of standard disks and less than a sentence under laboratory tests, and by a short discussion under composition and bacterial defects. Only the simplest laboratory procedures are described. There are few references, and the citations to Federal regulations are out of date.

The authors did not intend this book to be a laboratory manual, and its chief use to the chemist will be to assist him in the interpretation of analyses. In scope it is intermediate between the text books on ice cream and the general volumes on food or dairy technology.

WILLIAM HORWITZ