TUESDAY—MORNING SESSION

REPORT ON PROCESSED VEGETABLE PRODUCTS

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

A brief study has been made of the status of each of the tentative methods at present in the chapter on Processed Vegetable Products. The following is a summary of the results of the study together with a recommendation on each of the methods which are at present tentative.

35.3—Insoluble Solids

This method appears in all of the editions of the *Book of Methods* from the first one published in 1916. Before that, it appeared in almost the identical form in Bureau of Chemistry Bulletin 152, page 118, 1911. Collaborative results were given in that bulletin, page 121. Additional collaborative results were published the following year in Bureau of Chemistry Bulletin 162, page 127. In view of this early collaborative work and the many years during which the method has appeared in the *Book of Methods* without challenge, it is recommended* that it now be made official.

35.4—Soluble Solids

Making the method for insoluble solids official will automatically have the same effect on the method for soluble solids, since the latter consists merely of subtracting the insoluble solids from the total solids, the method for which is already official.

35.5—Specific Gravity (Tomato Products)

Bonney reported collaborative results on this method at the time that it was originally recommended for tentative adoption, in *This Journal*, **19**, 254 (1936). In view of these results and of the fact that this method is now in everyday use by manufacturers of tomato products, it is recommended* that this method be made official.

35.9—Sodium Chloride—Method II—(Rapid Method)

Collaborative results obtained with this method have been reported in *This Journal*, **23**, 353 (1940), **24**, 424 (1941), and **25**, 446 (1942). It was recommended for adoption as official, first action, in 1942. At the same time, one of the collaborators recommended a slight change in the method. This recommendation was merely to permit a catch weight of about five grams instead of an exact weight of five grams. Through what appears to have been an oversight, Committee C recommended that the collaborator's suggestion be incorporated in the method, but neglected to

* For report of Subcommittee C and action of the Association see *This Journal*, 33, 47 (1950).

approve the Referee's recommendation that the method be made official, first action. After seven years successful use of the method, it is now recommended* that it be made official.

35.18—Field Corn in Canned Mixtures of Field and Sweet Corn

Satisfactory collaborative results were given in the original report of this method in *This Journal*, **11**, 136 (1928). It has been used successfully a number of times since then. It is now recommended* that this method be made official.

35.20, 35.21—Volatile Fatty Acids in Vegetable Juices

These methods are official. However, it is my understanding that W. I. Patterson, the Referee on Decomposition in Foods, will recommend that the method for determining volatile acids given in **24.11** be dropped. Since there appears to be little need for the method for vegetable juices, it is recommended* that it be deleted from the chapter on Processed Vegetable Products as well.

35.22—Moisture in Dried Products

This method is tentative and no collaborative work appears to have been done with it. It has been reported as not generally applicable to all dried food products. It is therefore recommended that it be dropped. Since there is a need for methods for determining moisture in dried products, particularly by the industry, it is also recommended* that studies on this subject be continued.

Catalase

The method for measuring catalase activity did not appear in the sixth edition of the *Book of Methods*, but was adopted as tentative at the 1946 meeting and was published in *This Journal*, **30**, 76, 1947. Collaborative studies were conducted by the Associate Referee and the method was reported as sound (*Ibid*, **30**, 413, 1947). It is recommended* that the method be adopted as first action, and that studies be continued to make the method more rapid.

REPORT ON PEROXIDASE IN FROZEN VEGETABLES

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

In order to obtain data on the reproducibility of various methods of extraction and measurement of peroxidase activity in frozen vegetables, samples of fresh green peas, green asparagus, and spinach procured from the local commission market were prepared to represent unscalded, pro-

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 47 (1950).

perly scalded and over-scalded material. The peas were hulled and washed, the hulls saved separately; the spinach was trimmed and washed, and the asparagus was cut to 6½ in. spears and washed. The washed vegetables were scalded in small lots for 2 min. at room temperature, 180° and 212°F., respectively, chilled in running water, surface moisture removed, and then frozen on trays in air at 0°F. overnight. The frozen vegetables, after removal of excess ice, were ground in a food chopper in a cold room, then mixed and reground to obtain a homogeneous sample. These scalding conditions were selected to represent the usual ranges of peroxidase activity encountered in frozen vegetables. The frozen ground peas, pea hulls, asparagus, and spinach were then divided into three lots and submitted in sealed jars for analysis to two collaborators in addition to our laboratory. The collaborators were:

1. Philip Townsley, Division of Food Technology, Univ. of California.
2. H. J. Meuron, Chemist, San Francisco Station, Food & Drug Administration, Federal Security Agency.
3. M. P. Masure, Chemist, Vegetable Products Section, Vegetable Processing Division, Western Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U. S. Dept. Agr., Albany 6, California.

The samples were prepared on May 20–21, 1949, and analyses were made in our laboratory during July–September, 1949, and by the other two collaborators during August–September.

As pointed out in the first report,[1] a number of procedures of preparing vegetable extracts for analysis and a number of methods of peroxidase assay have been reported in the literature. A comparison of six methods of extraction and nine methods of analysis was planned originally. The original plan, however, was too comprehensive for the limited time available to this project, and it was reduced. In our laboratory, Mr. Townsley analyzed preparations obtained by one method of extraction by all procedures and then used one or two methods of assay for extracts prepared by the six procedures. In the other two collaborative laboratories, six methods of extraction and three methods of analysis were compared. The following procedures of preparing enzyme extracts for analysis were compared:

(1) Comminute duplicate 50-g portions of each vegetable sample in a Waring Blendor for 3 min. with about 1 g of calcium carbonate and 200 ml cold (precooled to 0°C.) 2% aqueous sodium-chloride soln. Filter the resulting extract thru a gauze-backed cotton milk filter to remove larger solid particles, decant filtrate to remove excess calcium carbonate, and filter again.

(2) Comminute duplicate 50-g portions of each vegetable sample in a Waring Blendor with 3.0 ml of citrate buffer of *p*H 4.5 per gram of tissue, for 3 min. at room temperature. The citrate buffer is prepared by dissolving 126 g citric acid in ca 800 ml H_2O, adding sufficient NaOH (ca 136 ml of 7.5 *N* NaOH) to adjust *p*H at nearly 1000 ml, and making up to 1000 ml. Filter the resulting extract as above.

(3) Comminute duplicate 50-g portions of each vegetable sample in citrate

[1] M. A. Joslyn, *This Journal*, 32, 296 (1949).

buffer at *p*H 4.5 as in 2 above, but first saturate mixture of buffer and vegetable with N_2 gas from cylinder of compressed gas, and displace all air in the blendor jar with N_2. Then blend for 3 min. with good flow of N_2 thru jar. Filter as above.

(4) Comminute duplicate 50-g portions of each vegetable sample in a Waring Blendor with 200 ml of 0.1 M phosphate buffer at *p*H 8. Filter thru milk filter to remove larger particles.

(5) Comminute duplicate 50-g portions of each vegetable sample with 200 ml of distilled water in a Waring Blendor for 3 min. Filter thru milk filter as above.

(6) Comminute duplicate 50 g portions of each vegetable sample with 200 ml of distilled water and 1 g of $CaCO_3$ in a Waring Blendor for 3 min. Filter thru milk filter, decant filtrate to remove excess calcium carbonate, and filter again.

The filtration was carried out without suction until the filter was dry. Excessively long filtration was avoided because of danger of surface denaturation of peroxidase. In our laboratory, since gauze-backed cotton milk filters were not available at first, the original runs were made using 8 layers of cheesecloth as filter medium. Subsequent tests indicated that these filtrates were similar in activity to those obtained with Johnson & Johnson single face rapid-flo filter disks. In expressing the results it was assumed that the vegetable tissue had a specific gravity of 1.0 and allowance was made for volume of tissue in calculating total volume of extract from volume of buffer added. On this basis each ml. of extract obtained by methods 1, 4, 5, and 6 would be equivalent to 0.2 g. of tissue; and each ml. of extract obtained by methods 2 and 3 would be equivalent to 0.25 g. of tissue.

The enzyme activity in terms of grams of vegetable tissue present in the aliquot of extract taken for analysis was determined from peroxidase activity measured as follows. These procedures, taken largely from the literature, represent the variations that exist at present.

1. COLORIMETRIC GUAIACOL PROCEDURE

(a) Add 2 ml of filtrate to 20 ml of water in a test tube (with raw or unblanched vegetables considerably less than 2 ml of filtrate should be used, in order to avoid too rapid development of color, but in all cases the total volume of water plus filtrate should be 22 ml). Then add 1.0 ml of 0.5% guaiacol in 50% alcohol down side of tube so as to form floating layer. Add 1.0 ml of 0.085% or 0.05 *N* H_2O_2 (2.38 ml of 30% reagent diluted to 1 liter) in same manner. Close top of tube, invert quickly 2 or 3 times to mix, and immediately pour off part into 14 mm Klett-Summerson colorimeter tube. Place tube in Klett-Summerson colorimeter previously adjusted to zero with tube of distilled water plus enzyme filtrate using the 420-millimicron filter. Bring galvanometer to balance point as quickly as possible, start stop-watch, and record colorimeter reading. Measure the rate of color formation over the first 5–10 min. and plot color intensity against time. From the linear portion of the resulting graph, calculate the delta colorimeter reading per min. per g. of tissue.

(b) 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 temp.

(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, and express activity as delta colorimeter reading per min. per g of tissue.

(c) Pipette 1 ml of 0.5% aqueous guaiacol and 1 ml of 0.1 *N* hydrogen peroxide into 20 ml of 0.01 *M* acetate buffer at *p*H 5.0 and 25°C. in an Evelyn colorimeter tube. Then add 1 ml of enzyme soln (or more or less enzyme soln with correspondingly less or more buffer, shake tube and place in colorimeter. Read colorimeter at intervals of 5–30 sec, depending on the rate of reaction, using a 420 millimicron filter. Plot values of 2-log T (T = % of light transmission) against time, and from linear portion obtain Δ log T units per min.

2. TITRIMETRIC PEROXIDE PROCEDURE

(a) *Modified Balls and Hale pyrogallol procedure*

Introduce 200 ml of distilled water and 25 ml of 0.2 *M* phosphate buffer *p*H 8.0 into a 300-ml stoppered flask, and bring to temperature in a water bath at 30°C.; add 2 ml of 0.2 *N* hydrogen peroxide and 10 ml of 6.25% pyrogallol soln and thoroly mix contents. Then introduce an aliquot of enzyme extract (1–10 ml) that does not decompose more than ⅓ of the hydrogen peroxide added and again mix contents of flask. Remove a 25-ml aliquot and place in a 125-ml flask containing 0.5 gm pyrogallol and 25 ml of 2 *N* sulfuric acid. Take time at half delivery of the pipette as the starting time of the run. Then add 10 ml of 10% potassium iodide and allow the flask to stand for 12–14 min. The liberated iodine is titrated with 0.01 *N* thiosulfate, using starch indicator. Take additional 25 ml aliquots at time intervals of 2, 5, 7, and 10 min. From the titrations calculate the peroxidase units from

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

where a = initial titration with 0.01 *N* thiosulfate at 0 mm in ml
x = titration at time t, ml
P.E. = peroxidase units in aliquot used for titration.

(b) *Guiacol procedure*

Bring to 25°C. 25 ml of 1 *M* acetate buffer (*p*H 5.6), 10 ml of 10% guiacol in 95% alochol, and sufficient water to make 250 ml when the enzyme extract (1–10 ml) and 5 ml of 0.75% H_2O_2 are added. Then add the enzyme extract and hydrogen peroxide. Immediately after adding the H_2O_2 thoroly mix and quickly remove a zero-time aliquot of the completed reaction mixture with a 25 ml rapid-flow pipette, and blow it into a 125-ml Erlenmeyer flask containing 25 ml 2 *N* sulfuric acid containing molybdate (55 ml conc. H_2SO_4, 0.1 g of finely-ground ammonium molybdate diluted to 1 liter). (Zero time is the time that delivery of the aliquot from the pipette is started.) Remove additional aliquots at 5 and 10 min. or other suitable intervals. At any time within an hour add to each flask 25 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 3–5 min, titrate excess thiosulfate with 0.01 *N* iodine, using about 10 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
x = iodine equivalent of residual H_2O_2 at time t

In both modifications it is preferable to calculate the results in terms of moles of peroxide used per min. per g of tissue. At 30°C., the P. E. value in (a) when multi-

plied by 0.00184 will give mg of purpurogallin produced by 1 mg of enzyme material in 5 min.

(c) Repeat (a) or (b), saturating the soln with N_2 gas and making the titrations under a blanket of N_2.

3. ASCORBIC ACID OXIDATION PROCEDURE

(a) With leuco 2,6-dichlorobenzenoneindophenol as substrate, pipette 1 ml of enzyme filtrate (for low enzyme activity blanched vegetables; for more active preparations dilute) into a 25-ml flask. Add 1 ml of ascorbic acid soln (50 mg of ascorbic acid dissolved in 100 ml of sodium oxalate-boric acid buffer. Prepare the buffer by disolving 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 3 ml of 2,6-dichlorphenolindophenol soln (prepared by dissolving 200 mg of dye in 100 ml warm distilled water, filtering and diluting to 1 liter at room temp). 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 0.5 ml of 0.3% H_2O_2 from a blow-out pipette, and start stop watch. Shake flask and return to water bath. After exactly 1 min., quickly add 1 ml of inhibiting acid (100 g metaphosphoric acid dissolved in cold water, filtered and brought to 1 liter). Titrate mixture with dye until pink color permanent for 30 sec. appears. Standardize dye soln against freshly prepared ascorbic acid (the dye soln should be stored in refrigerator and prepared weekly). Calculate the peroxidase value—number of mg of ascorbic acid oxidized in 1 min. by 1 gm 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 1 ml ascorbic acid − dye used for titration in ml plus dye added initially.

C = Conc. of dye in mg of ascorbic acid per ml.

g = g of enzyme-containing substance present in aliquot tested.

(b) With guaiacol as substrate, pipette 20 ml of 0.1 *M* oxalate—0.1 *M* phosphate buffer of *p*H 6.0, and 1 ml. of ascorbic acid soln (1 mg per ml)—into glass-stoppered 100 ml volumetric flask. Float flask on surface of water bath at 25°C, the tied glass stopper hanging over edge to hold it in place. When temperature equilibrium has been attained, add 1–2 ml of enzyme soln by means of calibrated rapid delivery pipette. (The quantity of enzyme added should be such that not more than one-half of the ascorbic acid present will be oxidized.) Then add 2–3 ml of 0.5% guaiacol soln, shake and follow by 1 ml of 0.1 *N* H_2O_2 soln. Shake and allow reaction to proceed for measured interval of time, usually 2 min, after which it is stopped by blowing in 3 ml of 1 *M* oxalic acid from a large-bore pipette. Make flask to volume with distilled water and measure residual ascorbic acid. A zero-time blank to measure the exact initial amount of ascorbic acid is run by mixing ascorbic acid, guaiacol, H_2O_2, and enzyme, and then immediately adding oxalic acid. The residual ascorbic acid may be measured by titrating an aliquot with standardized 2,6-dichlorophenolindophenol or by photoelectric colorimetric procedure. In the latter mix 4 ml of the ascorbic acid soln and 9 ml of dye and read residual dye in photoelectric colorimeter. Express results as in (a) above.

4. POTASSIUM IODIDE OXIDATION

Blend 25 g of vegetable tissue in Waring Blendor with 140 ml of buffer consisting of 20 ml of 2% soluble starch, 10 ml of 0.1 *N* thio-sulfate, 4.5 g of potassium iodide, and sufficient 0.2 *N* acetate buffer (*p*H 4.7) to make 1 liter. After sample is reduced to fine state of subdivision, filter thru milk filter. Squeeze cloth practically dry by

hand after the main bulk of the liquid flows thru. Add sufficient buffer mixture to bring filtrate to 200 ml. To a 50-ml aliquot of the well-mixed extract, measured in a graduated cylinder, add 1 ml of 0.9% H_2O_2 (prepared by diluting 30% reagent) from a quick-flowing blow-out type of graduated pipette. Start stop watch when pipette is half empty. Record the time in seconds for the appearance of blue-black color, or first appearance of change in color. The reciprocal of this time in sec. is a measure of enzyme activity.

DISCUSSION

The data obtained to date are summarized in Tables 1 to 4. The comparison of colorimetric procedures **1(a)** and **1(b)**, as shown in Table 1, indicates that procedure **1(a)** gave higher results than **1(b)** for scalded asparagus, scalded peas, scalded pea pods, and scalded spinach in the case of one collaborator (MPM), and consistently lower results in the case of another (PT). The results reported by the three collaborators are quite divergent, MPM reporting significantly lower results than HP. The data obtained by HJM indicate that the method of extraction had the greatest effect on peroxidase activity, greatest activity being obtained with method 1 for asparagus and spinach, and with method 4 for peas and pea pods. Apparently, while consistent results can be obtained by one collaborator, the reproducibility of the colorimeter results is poor.

With titrimetric procedure **2(b)**, difficulty was experienced in obtaining consistent results, the individual titrations occasionally decreasing with time instead of increasing. This was particularly true of the blanched samples whose peroxidase activity was low. The few data obtained by MPM were completely out of line. Fairly close agreement between duplicates was found by PT, who also reported that conducting the titrations in presence of N_2 gas did not materially change the results. The agreement between duplicates by the same collaborator and the agreement between collaborators was so poor that the effect of method of extraction was not too clear. In general, methods 1 and 4 gave the highest results. Method **2(a)** was not significantly different from method **2(b)**. The data in Table 2 were calculated over a 10-minute period, instead of 5 minutes, to reduce inconsistencies.

The ascorbic acid procedures, as shown in Table 3, on the whole were more reproducible, if the few results obtained by MPM are disregarded. With method **3(a)**, higher activity was found in asparagus blanched at 212° with extraction by method 2, in asparagus blanched at 180° with extraction 2 and 3, and in raw asparagus with extraction 5 and 6. With peas, higher results were obtained with extractions 1 for peas scalded at 212°F. and at 180°F.; and 1, 4, 5 with raw peas. Pea pods behaved somewhat like peas. With spinach, the methods of extraction had but little effect on activity. The citrate buffer, however, tended to give consistently higher results, and the end point was not as sharp. One of the collaborators (PT) reported that the results obtained with citrate buffers (extractions

TABLE 1.—*Colorimetric procedures for peroxidase measurement*

VEGETABLE	EXTRACTION	PROCEDURE	SCALE DIVISIONS PER MIN. PER GRAM TISSUE						
			H.J.M.		M.P.M.		P.T.		
Asparagus 212	1	1b	0.8	—	—	—	—	—	—
	2	1b	0.4	—	—	—	—	—	—
	3	1b	0.6	—	—	—	—	—	—
	4	1b	0.8	—	—	—	—	—	—
	5	1b	0.8	—	—	—	—	—	—
	6	1b	0.5	—	—	—	—	—	—
Asparagus 180	1	1a	—	—	30.0	—	2.2	1.3	—
	2	1a	—	—	20.4	—	—	—	—
	1	1b	47.6	45.4	16.0	—	35.3	33.4	—
	2	1b	24	25.6	—	—	—	—	—
	3	1b	27	26	—	—	—	—	—
	4	1b	8.0	7.5	—	—	—	—	—
	5	1b	16.0	15.8	—	—	—	—	—
	6	1b	15.8	14.2	—	—	—	—	—
Asparagus raw	1	1a	—	—	—	—	87.5	112	—
	1	1b	500	462	—	—	273	—	—
	2	1b	160	160	—	—	—	—	—
	3	1b	178	165	—	—	—	—	—
	4	1b	353	353	—	—	—	—	—
	5	1b	200	200	—	—	—	—	—
	6	1b	214	214	—	—	—	—	—
Peas 212	1	1a	—	—	117	—	0	0	—
	1	1b	11.5	12.0	56	—	2.8	3.7	—
	2	1b	5.1	4.8	—	—	—	—	—
	3	1b	5.4	5.4	—	—	—	—	—
	4	1b	15.0	15.0	—	—	—	—	—
	5	1b	<3	<3	—	—	—	—	—
	6	1b	<3	—	—	—	—	—	—
Peas 180	1	1a	—	—	48.3	—	18	—	—
	1	1b	100	100	13.3	—	61.5	—	—
	2	1a	—	—	17.3	—	—	—	—
	2	1b	25.6	27.6	4.3	—	—	—	—
	3	1b	17.5	16.7	—	—	—	—	—
	4	1b	77	91	—	—	—	—	—
	5	1b	40	37.5	—	—	—	—	—
	6	1b	37.5	37.5	—	—	—	—	—
Peas, raw	1	1a	—	—	—	—	1340	1880	1620
	1	1b	1765	1818	—	—	2500	2790	—
	2	1b	1334	1200	—	—	—	—	—
	3	1b	1334	1263	—	—	—	—	—
	4	1b	1875	1875	—	—	—	—	—
	5	1b	1579	1765	—	—	—	—	—
	6	1b	1765	2000	—	—	—	—	—

TABLE 1.—*Continued*

VEGETABLE	EXTRACTION	PROCEDURE	SCALE DIVISIONS PER MIN. PER GRAM TISSUE						
			H.J.M.		M.P.M.		P.T.		
Pea pods	1	1a	—	—	117	—	57.7	62.5	71.5
212	1	1b	454	435	56	—	300	300	250
	2	1a	—	—	78	—	—	—	—
	2	1b	166	160	55	—	—	—	—
	3	1b	61	64.5	—	—	—	—	—
	4	1b	500	500	—	—	—	—	—
	5	1b	200	176	—	—	—	—	—
	6	1b	238	227	—	—	—	—	—
Pea pods	1	1a	—	—	625	—	500	625	—
180	1	1b	1250	1250	314	—	1920	2000	—
	2	1b	480	462	—	—	—	—	—
	3	1b	606	666	—	—	—	—	—
	4	1b	1875	1765	—	—	—	—	—
	5	1b	536	577	—	—	—	—	—
	6	1b	667	625	—	—	—	—	—
Pea pods	1	1a	—	—	—	—	3600	—	—
raw	1	1b	4286	4000	—	—	8000	—	—
	2	1b	2000	2048	—	—	—	—	—
	3	1b	2526	2400	—	—	—	—	—
	4	1b	3529	3333	—	—	—	—	—
	5	1b	1071	1250	—	—	—	—	—
	6	1b	1875	1667	—	—	—	—	—
Spinach	1	1a	—	—	—	—	0	—	—
212	1	1b	2	—	—	—	0	—	—
	2	1b	<0.8	—	—	—	—	—	—
	3	1b	<0.4	—	—	—	—	—	—
	4	1b	0.8	—	—	—	—	—	—
	5	1b	0.0?	—	—	—	—	—	—
	6	1b	0.0?	—	—	—	—	—	—
Spinach	1	1a	—	—	5	—	—	—	—
180	1	1b	8.3	8.3	1	—	—	—	—
	2	1b	3.2	3.2	2.4	—	—	—	—
	3	1b	3.8	—	—	—	—	—	—
	4	1b	3.0	—	—	—	—	—	—
	5	1b	4.3	—	—	—	—	—	—
	6	1b	5.0	4.7	—	—	—	—	—
Spinach	1	1b	500	462	—	—	—	400	—
raw	2	1b	240	240	—	—	—	—	—
	3	1b	229	240	—	—	—	—	—
	4	1b	353	353	—	—	—	—	—
	5	1b	200	200	—	—	—	—	—
	6	1b	214	214	—	—	—	—	—

TABLE 2.—*Comparison of titrimetric procedures*

VEGETABLE	EXTRACTION	PROCEDURE	P.E. PER GRAM OF TISSUE					
			H.J.M.		M.P.M.		P.T.[a]	
Asparagus	1	2a	—	—	—	—	0.003	0.000
212	1	2b	—	—	—	—	0.0045	0.013
Asparagus	1	2a	—	—	—	—	0.015	0.010
180	1	2b	0.41	—	0.004	—	0.044	0.020
Asparagus	1	2a	—	—	—	—	0.015	0.32
raw	1	2b	0.41	0.51	—	—	0.32	0.26
	2	2b	0.44	0.26	—	—	—	—
	3	2b	0.09	0.18	—	—	—	—
	4	2b	0.22	0.24	—	—	—	—
	5	2b	0.24	0.34	—	—	—	—
	6	2b	0.24	0.21	—	—	—	—
Peas	1	2a	—	—	—	—	0.005	0.005
212	1	2b	0.00	0.00	0.005	—	0.10	0.000
	2	2b	−0.00	−0.00	—	—	—	—
Peas 180	1	2a	—	—	—	—	0.025	0.033
	1	2b	−0.00	0.05	—	—	0.031	0.030
	2	2b	0.00	0.00	—	—	—	—
Peas raw	1	2a	—	—	—	—	1.11	0.93
	1	2b	1.11	1.24	—	—	0.60	0.59
	2	2b	0.50	0.60	—	—	1.04	1.06
	3	2b	0.50	0.60	—	—	—	—
Pea pods	1	2a	—	—	—	—	0.035	0.035
212	1	2b	0.09	0.12	0.026	—	0.076	0.103
	2	2b	0.02	0.05	—	—	—	—
	3	2b	0.05	0.05	—	—	—	—
	4	2b	0.10	0.12	—	—	—	—
	5	2b	0.045	0.053	—	—	—	—
	6	2b	0.05	0.06	—	—	—	—
Pea pods							0.16	
180	1	2a	—	—	—	—	0.19	0.17
	1	2b	0.72	0.70	0.22	0.02	0.41	0.45
	2	2b	0.20	0.26	—	—	—	—
	3	2b	0.32	0.25	—	—	—	—
	4	2b	0.87	0.85	—	—	—	—
	5	2b	0.45	0.31	—	—	—	—
	6	2b	0.36	0.26	—	—	—	—
Pea pods	1	2a	—	—	—	—	0.67	0.54
raw	1	2b	2.42	2.24	—	—	1.68	1.75
	2	2b	1.24	1.15	—	—	—	—

TABLE 2.—*Continued*

VEGETABLE	EXTRACTION	PROCEDURE	P.E. PER GRAM OF TISSUE					
			H.J.M.		M.P.M.		P.T.*	
	3	2b	1.30	0.94	—	—	—	—
	4	2b	2.40	2.18	—	—	—	—
	5	2b	1.38	0.96	—	—	—	—
	6	2b	0.78	0.66	—	—	—	—
Spinach	1	2a	—	—	—	—	0.00	0.00
212	1	2b	—	—	—	—	0.004	0.003
Spinach	1	2a	—	—	—	—	0.004	0.039
180	1	2b	0.00	—	0.00	—	0.00	0.013
	4	2b	0.01	—	—	—	—	—
Spinach	1	2a	—	—	—	—	0.165	0.15
raw	1	2b	0.41	0.34	—	—	0.285	0.365
	2	2b	0.20	0.23	—	—	—	—
	3	2b	0.14	0.18	—	—	—	—
	4	2b	0.28	0.32	—	—	—	—
	5	2b	0.13	0.18	—	—	—	—
	6	2b	0.34	0.19	—	—	—	—

* Second figures for N_1.

2 and 3) markedly varied with speed of titration, being consistently lower with faster rates of titration. Ascorbic acid was oxidized faster by all vegetables in the presence of leuco indophenol than in presence of guaiacol.

The results obtained for the potassium iodide oxidation procedure by PT are shown in Table 4 in quadruplicate, and agree fairly well. A comparison between methods **1(b), 2(b), 3(a)** and **4**, for extraction by procedure 1 is shown in Table 5. This indicates that relative peroxidase activity for the same vegetable as determined by four procedures differs with the method used.

H. J. Meuron made the following comments on the three methods he tested:

"From the results of this work it appears that of the three methods used (reference your mimeographed instruction sheet) method **1(b)** seems to show the greatest variation in testing the extraction procedures. With this method, procedures 1 and 4 generally show the highest peroxidase activity, and the method at the same time produces closest agreement of duplicates. However, this method has the disadvantage of using colorimeter scale divisions as the unit of comparison, and since not all laboratories have the same type of colorimeter available, the units would not be directly comparable.

"The duplicate results from method **2(b)** vary so much that no valid conclusions can be drawn as to preference of method of extraction. I believe that my sample aliquots used in this method were too large, using up more than the desired amount

TABLE 3.—*Comparison of ascorbic acid procedures*

VEGETABLE	EXTRACTION	PROCEDURE	PEROXIDASE VALUE IN MG ASCORBIC ACID PER MIN PER GM OF TISSUE					
			H.J.M.		M.P.M.		P.T.	
Asparagus	1	3a	0.06	0.10	—	—	0.009	0.009
212	1	3b	—	—	—	—	0.00770	0.00770
	2	3a	0.19	0.16	—	—	—	—
	3	3a	0.10	0.10	—	—	—	—
	4	3a	0.10	0.10	—	—	—	—
	5	3a	0.05	0.05	—	—	—	—
	6	3a	0.03	0.03	—	—	—	—
Asparagus							0.73	0.73
180	1	3a	1.13	1.04	0.02	—	0.47	0.47
	1	3b	—	—	—	—	0.00574	0.00574
							2.09	1.40
	2	3a	1.18	1.25	0.42	—	2.09	1.40
	3	3a	1.38	1.46	—	—	1.7	2.1
	4	3a	0.78	0.78	—	—	0.42	0.42
	5	3a	0.69	0.69	—	—	0.28	0.28
	6	3a	0.78	0.69	—	—	0.28	0.28
Asparagus	1	3a	6.93	6.93	—	—	4.30	4.30
raw	1	3b	—	—	—	—	1.16	1.16
	2	3a	4.16	5.54	—	—	—	—
	3	3a	6.24	6.24	—	—	—	—
	4	3a	7.80	6.93	—	—	—	—
	5	3a	8.67	8.67	—	—	—	—
	6	3a	7.80	8.67	—	—	—	—
Peas 212	1	3a	0.40	0.40	—	—	0.13	0.13
212	1	3b	—	—	—	—	0.0407	0.0407
	2	3a	0.23	0.18	—	—	—	—
	3	3a	0.18	0.14	—	—	—	—
	4	3a	0.40	0.34	—	—	—	—
	5	3a	0.34	0.29	—	—	—	—
	6	3a	0.23	0.23	—	—	—	—
Peas							0.47	0.47
180	1	3a	1.71	1.43	0.04	—	0.39	0.39
	1	3b	—	—	—	—	0.0557	0.0557
	2	3a	0.34	0.34	0.00	—	0.54	0.54
	3	3a	0.57	0.46	—	—	0.59	0.59
	4	3a	0.71	0.71	—	—	0.16	0.16
	5	3a	0.71	0.57	—	—	0.19	0.19
	6	3a	0.86	0.57	—	—	0.25	0.25
Peas raw	1	3a	27.1	27.1	—	—	33.2	33.2
	1	3b	—	—	—	—	2.07	2.07
	2	3a	14.8	16.0	—	—	—	—
	3	3a	14.8	16.0	—	—	—	—
	4	3a	28.5	27.1	—	—	—	—

TABLE 3.—*Continued*

VEGETABLE	EXTRACTION	PROCEDURE	PEROXIDASE VALUE IN MG ASCORBIC ACID PER MIN PER GM OF TISSUE					
			H.J.M.		M.P.M.		P.T.	
	5	3a	32.8	34.2	—	—	—	—
	6	3a	24.2	22.8	—	—	—	—
Pea pods							2.3	2.3
212	1	3a	3.3	4.4	—	—	2.5	2.5
	1	3b	—	—	—	—	0.205	0.205
	2	3a	3.3	2.6	—	—	5.0	5.0
	3	3a	2.0	2.4	—	—	5.5	5.5
	4	3a	3.0	3.3	—	—	0.7	0.7
	5	3a	2.2	1.7	—	—	1.1	1.1
	6	3a	2.8	2.5	—	—	1.4	1.4
Pea pods							6.37	6.37
180	1	3a	—	—	0.04	—	6.25	6.25
	1	3b	—	—	—	—	0.736	0.753
	2	3a	—	—	1.62	—	14.9	14.9
	3	3a	—	—	—	—	15.7	15.7
	4	3a	—	—	—	—	10.1	10.1
	5	3a	—	—	—	—	3.1	3.1
	6	3a	—	—	—	—	3.4	3.4
Pea pods	1	3a	26.0	27.2	—	—	27.1	27.1
raw	1	3b	—	—	—	—	3.57	3.57
	2	3a	18.7	20.0	—	—	—	—
	3	3a	17.0	17.9	—	—	—	—
Spinach	1	3a	0.31	0.23	—	—	0.13	0.13
212	1	3b	—	—	—	—	0.0030	0.0030
	2	3a	0.21	0.21	—	—	—	—
	3	3a	0.21	0.13	—	—	—	—
	4	3a	0.26	0.26	—	—	—	—
	5	3a	0.16	0.18	—	—	—	—
	6	3a	0.26	0.23	—	—	—	—
Spinach	1	3a	0.78	0.78	0.79	—	0.25	0.25
180	1	3b	—	—	—	—	0.0055	0.0055
	2	3a	0.42	0.49	0.00	—	—	—
	3	3a	0.55	0.55	—	—	—	—
	4	3a	0.78	0.69	—	—	—	—
	5	3a	0.61	0.61	—	—	—	—
	6	3a	0.78	0.78	—	—	—	—
Spinach	1	3a	15.6	17.3	—	—	9.64	11.1
raw	1	3b	—	—	—	—	0.207	0.207
	2	3a	11.1	11.1	—	—	—	—
	3	3a	9.0	10.4	—	—	—	—
	4	3a	14.7	13.9	—	—	—	—
	5	3a	10.4	10.4	—	—	—	—

TABLE 4.—*Potassium iodide oxidation procedure*

VEGETABLE	TIME IN SECONDS FOR APPEARANCE OF STARCH-IODIDE COLOR			
Asparagus				
212	357	363	352	358
180	277	291	281	274
raw	86	83	79	77
Peas				
212	295	290	285	284
180	288	290	278	282
raw	48	45	44	44
Pea pods				
212	180	177	182	175
180	102	106	106	109
raw	32	33	32	33
Spinach				
212	350	351	355	358
180	355	341	339	365
raw	279	281	287	243

TABLE 5.—*Comparison of methods of measuring peroxidase activity*

PRODUCT	METHOD OF ANALYSIS			
	1b	2b	3a	4
Asparagus				
raw	480	0.45	6.93	81
180	46	0.40	1.09	280
212	1	0.005	0.08	357
Peas				
raw	1780	1.16	27.1	44
180	100	0.00	1.58	285
212	12	0.00	0.40	289
Pea pods				
raw	4140	2.33	26.6	33
180	470	0.71	6.4	106
212	450	0.10	3.6	178
Spinach				
raw	480	0.37	16.4	285
180	8	0.00	0.78	350
212	2	0.004	0.27	354

of peroxide. However, even if duplicates agree closely, I would prefer methods 1(b) or 3(a) because the procedure used in method 2(b) is too involved.

"Method 3(a) uses a unit of activity (*i.e.*, mg of ascorbic acid/min./gram) which can be reproduced in any laboratory, and has this advantage over method 1(b). The procedure is also simple and fairly rapid. However, the difficulty of manipulation of a 25 ml flask and the use of such small quantities of reagents perhaps causes an abnormal magnification of slight variations when the results are calculated. Perhaps use of larger quantities of reagents would remove this factor. A difference in titration of 0.05 ml dye makes a difference of 10% in the calculated activity, yet 0.05 ml is as close as the titration can normally be made. This method is preferred over the other two wherever applicable.

"Helen Hyde of this laboratory tested the asparagus and spinach, using method 3(a) and the above comment on this method is submitted by her. She also assisted with method 2(b) on asparagus and spinach, and prepared the extracts of these vegetables.

"The Western Regional Research Laboratory of the U.S. Department of Agriculture very kindly loaned the colorimeter used in method 1(b).

"All results are calculated as decided upon at our conference of 13 September. I believe, however, that the results of method 2(b) would appear in a more favorable light if the titration difference $t_{10}-t_0$ were used, rather than that of $t_{10}-t_5$, since any error at t_5 will not reflect at t_{10}."

In view of the results obtained it is recommended that these procedures be studied further and in more laboratories.

REPORT ON COLORING MATTERS IN FOODS

By C. F. JABLONSKI (Food and Drug Administration, Federal Security Agency, New York 14, N. Y.), *Referee*

Following the request of the committee of the A.O.A.C. the Referee sent out several sets of dye samples to a number of collaborators. Each set consisted of four subdivisions which were marked No. 1, No. 2, No. 3, and No. 4, respectively. The coloring matter consisted of various amounts of Tartrazine (FD&C Yellow No. 5) and Sunset Yellow FCF (FD&C Yellow No. 6). The method of procedure submitted to the collaborators was modified somewhat from the one previously proposed.

It was as follows:

Quantitative Estimation of Tartrazine (FD&C Yellow No. 5) in presence of Sunset Yellow FCF (FD&C Yellow No. 6).

The method is based on the observation that Sunset Yellow FCF (FD&C Yellow No. 6) can be completely destroyed by hydrogen sulphid (H_2S) heated under pressure with the formation of a colorless solution. Tartrazine (FD&C Yellow No. 5) on the other hand, being more resistant to this treatment, is affected only to a slight degree. Since the amount of Tartrazine destroyed appears to be proportional under definite conditions, results of a quantitative order are obtained by applying a correction factor. Details of the method will be published in the 7th Ed. *Methods of Analysis* (1950).

The composition of the samples as deduced by TiCl titer were as follows:

	per cent
Sample No. 1—Tartrazine (FD&C Yellow No. 5)	22.27
Sunset Yellow FCF (FD&C Yellow No. 6)	66.18
Total color	88.45
Sample No. 2—Tartrazine (FD&C Yellow No. 5)	89.07
Sample No. 3—Sunset Yellow FCF (FD&C Yellow No. 6)	88.24
Sample No. 4—Tartrazine (FD&C Yellow No. 5)	66.81
Sunset Yellow FCF (FD&C Yellow No. 6)	22.06
Total color	88.87

The reports of the collaborators as received are as follows:

Qualitative

M. L. Offut — Sample No. 1—Contains Tartrazine and Sunset Yellow FCF
Sample No. 2—Contains Tartrazine

N.Y. District — Sample No. 3—Contains Sunset Yellow FCF
Sample No. 4—Contains Tartrazine and Sunset Yellow FCF

Quantitative

	per cent
Sample No. 1—Tartrazine (FD&C Yellow No. 5)	22.27
Sunset Yellow FCF (FD&C Yellow No. 6)	66.38
Total color	88.65
Sample No. 2—Tartrazine (FD&C Yellow No. 5)	88.21
Sample No. 3—Sunset Yellow FCF (FD&C Yellow No. 6)	87.90
Sample No. 4—Tartrazine (FD&C Yellow No. 5)	67.13
Sunset Yellow FCF (FD&C Yellow No. 6)	21.14
Total color	88.27

Qualitative

Dr. R. C. Cooney — Sample No. 1—Contains Tartrazine and Sunset FCF Yellow
H. Kohnstamm & Co. Sample No. 2—Contains Tartrazine
Sample No. 3—Contains Sunset Yellow FCF
Sample No. 4—Contains Tartrazine and Sunset Yellow

Quantitative

	per cent
Sample No. 1—Tartrazine (FD&C Yellow No. 5)	22.20
Sunset Yellow FCF (FD&C Yellow No. 6)	65.60
Total color	87.80
Sample No. 2—Tartrazine (FD&C Yellow No. 5)	89.20
Sample No. 3—Sunset Yellow FCF (FD&C Yellow No. 6)	88.60
Sample No. 4—Tartrazine (FD&C Yellow No. 5)	67.25
Sunset Yellow FCF (FD&C Yellow No. 6)	21.00
Total color	88.25

Qualitative

Fred J. Hope	Sample No. 1—Contains Tartrazine and Sunset Yellow FCF
H. Kohnstamm & Co.	Sample No. 2—Contains Tartrazine
	Sample No. 3—Contains Sunset Yellow FCF
	Sample No. 4—Contains Tartrazine and Sunset Yellow FCF

Quantitative

	per cent
Sample No. 1—Tartrazine (FD&C Yellow No. 5)	23.38
Sunset Yellow FCF (FD&C Yellow No. 6)	65.85
Total color	89.23
Sample No. 2—Tartrazine (FD&C Yellow No. 5)	89.31
Sample No. 3—Sunset Yellow FCF (FD&C Yellow No. 6)	88.97
Sample No. 4—Tartrazine (FD&C Yellow No. 5)	63.60
Sunset Yellow FCF (FD&C Yellow No. 6)	25.89
Total color	89.49

Qualitative

I. Hanig	Sample No. 1—Contains Tartrazine and Sunset Yellow FCF
H. Kohnstamm & Co.	Sample No. 2—Contains Tartrazine
	Sample No. 3—Contains Sunset Yellow FCF
	Sample No. 4—Contains Tartrazine and Sunset Yellow FCF

Quantitative

	per cent
Sample No. 1—Tartrazine (FD&C Yellow No. 5)	22.79
Sunset Yellow FCF (FD&C Yellow No. 6)	66.01
Total color	88.80
Sample No. 2—Tartrazine (FD&C Yellow No. 5)	88.92
Sample No. 3—Sunset Yellow FCF (FD&C Yellow No. 6)	88.84
Sample No. 4—Tartrazine (FD&C Yellow No. 5)	63.80
Sunset Yellow FCF (FD&C Yellow No. 6)	25.14
Total color	88.94

Qualitative

J. L. Hogan	Sample No. 1—Contains Tartrazine and Sunset Yellow
	Sample No. 2—Contains Tartrazine
	Sample No. 3—Contains Sunset Yellow
	Sample No. 4—Contains Tartrazine and Sunset Yellow

Quantitative

	per cent
Sample No. 1—Tartrazine (FD&C Yellow No. 5)	22.38
Sunset Yellow (FD&C Yellow No. 6)	63.22
Total color	85.60

Sample No. 2—Tartrazine (FD&C Yellow No. 5)	86.77
Sample No. 3—Sunset Yellow (FD&C Yellow No. 6)	86.77
Sample No. 4—Tartrazine (FD&C Yellow No. 5)	65.13
Sunset Yellow (FD&C Yellow No. 6)	21.31
Total color	86.50

The comments of the collaborators were as follows:

M. L. Offutt—No difficulty encountered in method.
R. C. Cooney—No comments.
J. L. Hogan—No comments.
Fred J. Hope—No difficulty in performing these analyses.
I. Hanig—The analytical method presented no difficulties.

DISCUSSION

The results submitted by the collaborators can be considered quite satisfactory, particularly the values reported for samples No. 1, No. 2, and No. 3, which are almost identical. However, the figures obtained for sample No. 4 show a greater diversion since the extremes extend to 3%.

On this point the Referee is of the opinion that the discrepancies may be due to a varied interval between the period of cooling (after the autoclave digestion) and the ultimate titration. It has been experimentally established that a slight degradation of FD&C Yellow No. 5 is continuously proceeding under the specified conditions.

The Referee, therefore, suggests that a time factor be set for titration after autoclave treatment. A reasonable interval after 1 hour heat and pressure treatment and permitting cooling to proceed in the autoclave should not exceed 4–5 hours, after which the titrations should be carried out. By performing the operations in reasonably definite time periods, results of high accuracy are attainable. The Referee therefore recommends that a time limit be included in the method.

RECOMMENDATIONS*

It is recommended—

(1) That study of the method for the quantitative estimation of Tartrazine (FD&C Yellow No. 5) in presence of Sunset Yellow FCF (FD&C Yellow No. 6) be continued.

(2) That investigational work be undertaken to separate and determine quantitatively Sunset Yellow FCF (FD&C Yellow No. 6) in presence of Amaranth (FD&C Red No. 2).

(3) That investigational work be undertaken to separate and determine quantitatively Light Green S. F. Yellowish (FD&C Green No. 2), Fast Green FCF (FD&C Green No. 3), and Brilliant Blue FCF (FD&C Blue No. 1).

(4) That investigational work be undertaken to separate quantita-

* For report of Subcommittee C and action of The Association, see *This Journal*, 33, 79 (1950).

tively yellow AB (FD&C Yellow No. 3), Yellow OB (FD&C Yellow No. 4), Orange SS (FD&C Orange No. 2), and Oil Red XO (FD&C Red No. 32).

(5) That collaborative work on analytical methods for coal-tar colors certifiable for use in foods be conducted.

REPORT ON COFFEE AND TEA

By H. A. LEPPER (Division of Food,* Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Referee*

A review of the methods in Chapter 18 of *Methods of Analysis* has been made with a view to the reclassification of the methods in keeping with that to be employed in the forthcoming 7th revision. In the recommendations which follow, the section numbers refer to the 6th edition.

RECOMMENDATIONS†

COFFEE

18.1, 18.3—Macroscopic Examination (Green and Roasted Coffees), Coloring Matters in Green Coffee

These tentative methods should be retained as procedures.

18.5—Moisture

Although tentative, this method is the usual oven-drying procedure applicable to foods in general and practically the same as that given in Bureau of Chemistry Bulletin 107, where it first appeared as applied to coffee. It provides for drying both under vacuum and in air. No record of collaborative study could be found, but inasmuch as it is a method of general principle in use since before 1908, it should be adopted as official.

18.6—Soluble Solids

This method has been referred to from time to time as "Winton's." It first appeared in A.O.A.C. proceedings in Lythgoe's referee report in B. C. Bulletin 90 page 42 (1904 meeting) and was called "cold water extract." It was one of a number of methods applied by Lythgoe to numerous samples of coffee. They were adopted (*Ibid.*, p. 226) without any record of collaborative study. The one on soluble solids has been the only one used since that time and should be made official.

18.14–18.15—Caffeine

There are three methods for caffeine. The official Power-Chestnut was

* W. B. White, Chief.
† For report of Subcommittee C and action of The Association, see *This Journal*, 33, 47 (1950).

originally designed as one of application to vegetable products in general. It can be employed on caffeine-bearing leaves, seeds, or other plant materials. It is official for tea. The Association should have a method of such wide applicability and the Coffee chapter is the logical place for it. The Fendler-Stüber method was tentative for a number of years and made official only last year (*This Journal*, **32**, 84 (1949)). It was shown in 1921 (*Ibid.*, **4**, 526) that there is an inherent error in the Fendler-Stüber method due to the action of the potassium permanganate, used for purification, on the caffeine, and its deletion is recommended. The Bailey-Andrew method has been official for caffeine in tea for a number of years. It was studied collaboratively on dry coffee extracts (*Ibid.*, **29**, 37 (1946)) and adopted as official, first action, for coffee (*Ibid.*, **30**, 70 (1947)). The method works equally well with green, roasted, and so-called decaffeinated coffees. It is recommended that it be made official and the following direction given under Coffee: "Proceed as under **18.41** using 10 g of sample prepared as in **18.4.**"

18.17—Starch

The present method involves application of the diastase procedure. Originally Lythgoe (Bulletin 90) proposed both an acid hydrolysis and a diastase conversion of the starch before the determination of the reducing sugars formed. These appeared in Bulletin 107 as "Crude Starch by Direct Inversion" and "Starch," respectively. The latter is the present starch method. There is no record of collaborative study on coffee. The method after preliminary solution preparation refers by number to the official diastase starch method under grains. There is no starch in coffee. Inasmuch as the determination is to furnish a basis for the estimation of starch-bearing adulterants identified by other means if present (which are usually the cereals), the method which is official for cereals should be official for coffee.

18.18—Sugars

This is the usual Munson-Walker reducing sugar method wherein ammonium sodium phosphate is used as the clarifying agent to eliminate interfering magnesium. It was proposed in *This Journal*, **3**, 498 (1920). There was so little interest in these determinations in coffee that collaboration could not be obtained. This method employs the general principles of the official method for determining sugars in vegetable products. The coffee method should be official also.

18.20—Total Acid

This is a simple, direct titration on which collaboration would hardly appear necessary. It should be official.

18.21—Volatile Acidity

This determination has little or no application. The Association will no doubt adopt more accurate and modern methods for volatile acids in food products. If need arises for the determination in coffee, their adaptability can be studied. In the meantime this method should be dropped.

18.22—Sugar and Dextrin

18.23—Egg Albumen and Gelatine

18.25—Fats and Waxes

These three methods should be continued as procedures.

18.24—Chickory Infusion

This method should be retained as a procedure but should not be included as at present under the section on "Coating and Glazing Substances."

TEA

18.26—Dust, Stems, and Foreign Leaves

This tentative method should be retained as a procedure.

18.38—Protein

This tentative method involves a simple determination of nitrogen by the Kjeldahl procedure, or its established variations, which has been official for all types of products for many years. The total nitrogen is corrected for the nitrogen due to caffeine which is determined by an official method. Protein is then calculated by use of a factor and the procedure should be made official.

18.42–18.43—Tannin

The reference on this method indicates that it dates back to Bureau of Chemistry Bulletin 13, Part 7. It appears to be one of the oldest methods applied to the analysis of tea. It now has little application or use. It was originally of value when there were a number of false claims made on teas on the market of low tannin content. It is believed that the method should be retained in case occasion should arise requiring a determination of tannin in tea and, because of its well-established standing through many years of recognition, should be made official.

18.44–18.46—Facing

These tentative methods should remain as procedures.

CHLOROGENIC ACID IN COFFEE

Recently there has come to the attention of regulatory officials some advertising and label claims that certain coffees on the market are supe-

rior because of freedom from bitter taste due to a reduced chlorogenic content. For many years the bitterness of some coffees was associated with the acids present, and at one time they were referred to as caffetannic acid. The tannins and tannic acids have long been associated with bitterness. At one time claims similar to those now being made appeared in connection with the marketing of coffee, except that caffetannic acid was the constituent supposed to have been reduced. To check the falsity of these claims, it is recommended that methods for chlorogenic acid should be developed.

REPORT ON DAIRY PRODUCTS

By WILLIAM HORWITZ (Food and Drug Administration, Federal Security Agency, Minneapolis 1, Minn.), *Referee*

The dairy chapter of the *Book of Methods* has been reviewed to determine the future status of those procedures now marked "Tentative." Those tentative methods on which collaborative work has been performed are recommended for adoption as "First Action." The well-known methods which have become accepted procedures through long usage, even though they may not have been subjected to collaborative work, are also recommended as first action. Qualitative tests, sampling, and preparation of sample paragraphs are recommended as "Procedures." For brevity these recommendations are summarized in Table 1, together with references to collaborative work as published in *This Journal* and brief remarks supporting the recommendation where pertinent. Changes which may be considered editorial in nature have not been discussed. Some minor actions which should be taken are: **22.22,** Lactose—Polarimetric Method, has been in a first action status and should now be made official. The statement "dull red heat" in method **22.99,** Ash (Dried Milk) should be changed to "550°C" to conform with the wording of the identical method for fluid milk.

Fat.—As indicated by numerous inquiries there is a fairly widespread misinterpretation of the sentence in the Roese-Gottlieb method for fat, **22.25,** "Centrifuge Mojonnier flask 20 min. at ca 600 r.p.m. or let it (or Rohrig tube) stand until the upper liquid is practically clear." The option of centrifuging the flask was inserted into the 6th edition of *Methods of Analysis,* following the work of Steagall[1] who showed that centrifuging effectively breaks emulsions and eliminates the waiting period. These emulsions, however, are usually encountered with evaporated milk and are rarely troublesome with a product such as cheese. The important requirement of the above sentence is that the mixed ether layer be "practically clear," and whether this is achieved by centrifuging

[1] *This Journal,* **28,** 207 (1945).

or by standing, or even by a combination of these two techniques, is at the option of the operator.

Muers and House[2] have recently shown that the use of ether contaminated with peroxide in the Roese-Gottlieb method for fat in dairy products may lead to values that are too high by amounts up to 2% of the fat figure. Additional data on this point will be collected by the Referee during the coming year to support their contention.

The Associate Referee, acting as chairman of a subcommittee of the American Dairy Science Association, recommends the adoption of a modified Babcock procedure which uses a slightly larger volume of milk at higher than room temperature and eliminates the meniscus with a colored mineral oil. The purpose of the modification is to make the results by the Babcock test agree with those obtained with the Roese-Gottlieb procedure. The Babcock test is probably the most widely used procedure in the *Book of Methods* and is fundamental to the present system of paying for milk and cream according to fat content. On the previous occasion of the revision of the Babcock procedure the Associate Referee sent a letter to a number of individuals interested in the test outlining the proposed changes and requesting comments,[3] and the replies were considered in the recommendations. It is the Referee's opinion that the changes recommended are of such a nature that all interested parties be requested to submit their comments and data to the Associate Referee for consideration.

Preparation of Butter Samples.—The new Associate Referee has inaugurated a study on the use of mechanical shaking and stirring devices and of artificial means of cooling the samples during preparation, and he intends to perform collaborative studies during the forthcoming year. He, as well as the previous Associate Referee, have recommended that tentative method **22.109,** Mechanical Stirrer Method of Preparation of Butter Samples, be dropped, and this recommendation is incorporated into Table 1.

Frozen Desserts.—The Associate Referee has reviewed the literature on this subject and intends to further investigate the fat and solids methods. He has recommended that the *Weight per Unit Volume* method be raised to first action status and has suggested several editorial changes in other methods.

Phosphatase.—A statistical study of three phosphatase tests indicates that the Sanders-Sager test is superior to the two that now appear in the *Book of Methods.* The Long Test (Gilcreas-Davis) has certain advantages when applied to a large number of samples simultaneously and is therefore recommended as first action, as applied to milk and cream only. The Rapid Test (Scharer laboratory) is not outstanding in any respect and the previous action to delete it should be unchanged.

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[2] *Analyst,* 74, 85 (1949).
[3] *This Journal,* 8, 471 (1924).

TABLE 1.—*Recommended action on tentative methods of the sixth edition of the "Book of Methods, A.O.A.C."*

METHOD	TITLE	RECOMMENDED ACTION	COLLAB. WORK REFERENCE	REMARKS
22.1	Collection of Sample	Procedure		
22.2	Preparation of Sample	Procedure		
22.3	Specific Gravity	Procedure	None	Physical measurement of a physical constant
22.4	Acidity	First Action	None	Widely used; currently being studied
22.5–.7	Citric Acid	First Action	16, 427 (1933)	Held in tent. status pending study on a newer method now being studied by Assoc. Referee on fruit acids
22.15	Total Solids (Approximate)	Procedure	None	Physical method similar to 22.3 but with a more accurate hydrometer
22.16	Ash	First Action	23, 453 (1940)	
22.19–.20	Casein, Method II	First Action	16, 489 (1933) 17, 357 (1934)	Held in tent. status pending a study as which of the two casein methods should be the single official method
22.36–.38	Hypochlorites and Chloramines	Procedure	None	Qualitative Test
22.40–.42	Sediment Test	Transferred to Extraneous Material Chapter	32, 54 (1949)	
22.59	Collection of Sample, Cream	Procedure		
22.60	Preparation of Sample, Cream	Procedure		
22.73	Preparation of Sample, Evaporated Milk	Procedure		
22.85	Preparation of Sample, Condensed Sweetened Milk	Procedure		
22.94	Sampling Dried Milk	Procedure		
22.95	Preparation of Sample	Procedure		

22.96	Moisture	First Action	11, 289 (1928) 10, 308 (1927)	
22.97	Protein	Official	None	Identical procedure official for fluid milk and many other products
22.98	Casein in Malted Milk and Chocolate Malted Milk	First Action	24, 545 (1941)	
22.99	Ash	First Action		Identical procedure recommended first action for fluid milk
22.101	Fat in Malted Milk	First Action	5, 508 (1922)	Held in tent. status pending development of a single method for malted and dried milk
22.104	Citric Acid in Dried Milk	First Action	16, 427 (1933)	See 22.5–22.7
22.106	Microscopic identification of Malted Milk and its Flavored Products	Procedure		
22.107	Butter Sampling	Procedure		
22.109–.110	Preparation of Sample Mechanical Stirrer Method	Delete		Present and previous Assoc. Referee recommendation
22.121–.122	Renovated Butter and Oleomargarine	Delete		Obsolete
22.123	Selection and Preparation of Sample	Procedure		
22.129	Cheese, Coloring Matter	Drop Tentative	None	Satisfactory procedure; Refer to Revision committee
22.132	Tartaric Acid, Qualitative Test	Procedure		
22.135	Citric Acid, Qualitative Test	Procedure		
22.137	Lactose in Process Cheese	Official	16, 485 (1933)	
22.138–.141	Gums in Soft Curd Cheese	First Action	28, 601 (1945)	Referee on gums concurs
22.143–.145	Ice Cream and Frozen Desserts—Weight per Unit of Packaged Ice Cream	Official	28, 601 (1945)	
22.146	Preparation of Sample, Ice Cream	Procedure		

RECOMMENDATIONS*

It is recommended—

(1) That the recommendations summarized in Table 1 for present tentative methods be adopted as indicated in that table.

(2) That method **22.23–.24,** Lactose—Polarimetric Method, be made official.

(3) That the statement "dull red heat" in method **22.99,** Ash in Dried Milk, be changed to "550°C."

(4) That the Associate Referee for Fat in Dairy Products inform interested and affected parties of the proposed changes in the Babcock procedure and review the comments and data submitted.

(5) That the following changes be made in **22.25** as a clarification of the directions: Line 6, delete "20 min. at ca 600 r.p.m." and add after the word "clear," line 7, "(Some dairy products may require centrifuging for as long as 20 min. at 600 r.p.m. for complete separation of emulsion.)"

(6) That studies be continued on the following subjects:

(a) Acidity of milk and ash in milk.

(b) Preparation of butter samples.

(c) The Babcock test with particular reference to homogenized milk and chococlate drinks.

(d) The serum methods for added water in milk.

(e) Sampling hard and soft cheeses.

(f) Reconstituted milk.

(g) Frozen desserts.

(h) Phosphatase test.

(7) (a) That the Sanders-Sager method for the detection of residual phosphatase be adopted as official for milk and cream, cheddar type cheese, and soft uncured cheeses (cottage, cream, etc.) and as first action, for all other dairy products.

(b) That the minor changes in the Sanders-Sager method be adopted as suggested by Associate Referee.

(c) That the Long Test be adopted as first action, applicable only to fluid milk and cream, together with an alternative photometric procedure.

(d) That the Rapid Test, **22.49–22.57,** be dropped.

(8) That the change in the method for fat in cheese "add a few glass beads . . . " recommended last year, be adopted as official.

(9) That the sour serum method (**22.29**) for added water be dropped.

* For report of Subcommittee C and action of the Association, see *This Journal,* 33, 48 (1950).

REPORT ON PHOSPHATASE TEST IN DAIRY PRODUCTS

By WILLIAM HORWITZ (*Associate Referee*), LILA F. KNUDSEN, and WILLIAM WEISS (U. S. Food and Drug Administration, Federal Security Agency, Minneapolis 1, Minnesota, and Washington 25, D. C.)

At the 1947 meeting the Association deleted the Gilcreas-Davis (long) and the Scharer (rapid) laboratory phosphatase tests (sections **22.43–22.57**) and substituted the Sanders-Sager procedure.[1] This method was made first action for fluid milk and cream, cheddar type cheese, and soft uncured cheeses, and tentative for other types of cheese and various other dairy products. Collaborative work had previously been performed on cheddar type and soft uncured cheeses.[2] Since the deleted methods were so widely used by Public Health Officials in the routine testing of milk for pasteurization, Committee C, at the 1948 meeting, recommended that these methods be re-evaluated and compared with the Sanders-Sager method by conducting collaborative work on milk or cream.

DETAILED STATEMENT OF PROBLEM

Eight methods are involved. These are four under the Sanders-Sager procedure, differing only in the manner in which the final colored reaction product is measured—by comparison with previously prepared color standards or by the use of a photometer—in both cases in either an aqueous or butyl alcohol phase (aqueous photometric reading, butyl alcohol photometric reading, aqueous color standards reading, and butyl alcohol color standards reading), and two under each of the Gilcreas-Davis and Scharer methods (the photometric and the color standards measurements).

It was desired to see which of these eight procedures was the best in regard to:

(a) *Lack of overlapping* of the results of any one analyst between the levels of raw milk chosen for use in the samples. (Whether there is overlapping of the results between 0% and 0.1% raw milk, between 0.1% and 0.4% raw milk, and between 0.4% and 1% raw milk.)

(b) *Linearity of relationship* between results of test and actual known percentage of raw milk for the several levels.

(c) *Specificity*—whether or not the reading on samples containing no raw milk is practically zero.

(d) *Reproducibility*—how closely on the average the results obtained by one analyst could check results obtained by another analyst at any other laboratory.

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[1] *This Journal*, 31, 82 (1948).
[2] *Ibid.*, 30, 422, 430 (1947).

DESIGN OF THE COLLABORATIVE EXPERIMENT

In order to properly evaluate the methods so that no one laboratory's results would be overemphasized and the results on no one method biased because of lack of collaboration on certain levels of underpasteurization, a statistical design was set up wherein two duplicate samples were run in duplicate by one of two analysts at each laboratory while only one of the duplicate samples was run in duplicate by the second analyst at that laboratory. Four levels of phosphatase (% of raw milk in the sample) were used: 0%, 0.1%, 0.4%, and 1% raw milk.

TABLE 1.—*Illustration of the design of the collaborative experiment*

	LEVELS OF RAW MILK							
	0%		0.1%		0.4%		1.0%	
	SAMPLE 1	SAMPLE 2	SAMPLE 1	SAMPLE 2	SAMPLE 1	SAMPLE 2	SAMPLE 1	SAMPLE 2
Lab. 1								
Analyst 1	X	X	X		X		X	X
Analyst 2	X		X	X	X	X	X	
Lab. 2								
Analyst 1	X	X	X	X	X		X	
Analyst 2	X		X		X	X	X	X
Lab. 3								
Analyst 1	X	X	X		X	X	X	
Analyst 2	X		X	X	X		X	X

Two duplicate samples were made on each level of phosphatase (making eight "samples" in all) and two analysts ran the analyses at each laboratory. However, since it was necessary to have a limit of six samples run by one analyst on one method, a statistically balanced design was used as illustrated in Table 1. This design is duplicated for each multiple of 3 laboratories.

The Sanders-Sager procedure was run by all laboratories, with a choice of running either or both of the other two methods on the same samples by the photometric or color standards modifications of reading the developed color. The samples of milk were prepared by reheating a commercially pasteurized skim milk to 82°C and cooling. This milk was used as a diluent for the preparation of 0.1%, 0.4%, and 1.0% raw whole milk concentrations. Skim milk was used as the diluting fluid to reduce possible complications from fat separation. A series of eight sample tubes were prepared for each laboratory, two from each of the raw milk dilutions and two from the reheated skim milk. These tubes were labeled by scrambling the letters A to H: samples E and F contained

the 0% raw milk; samples B and G, 0.1% raw milk; samples H and C, 0.4% raw milk; and samples A and D, 1.0% raw milk. All eight samples were sent to each laboratory with instructions that one analyst was to run a certain combination of six samples as specified by the design in Table 1, and the other analyst was to run a different combination of six samples. Samples F, B, H, and A were run by all analysts while the other samples were run by half of them, in such a combination that in each group of three laboratories the first group of samples (F, B, H, A) were run 48 times including duplicates, while the second set (E, G, C, D) were run 24 times.

Samples were preserved with chloroform and were shipped by air mail so that all collaborators could start determinations at the same time, as instructed in the letter of transmittal.

STATISTICAL ANALYSIS OF THE DATA

Altogether there were 1,176 individual determinations that were reported and used in the analysis. The results from two of the 20 laboratories participating were rejected (numbers 12 and 23) in the first case because the proper photometric filter was not available (which would result in a reduced sensitivity), and in the second case because the individual values for duplicate determinations were not available. Although the results from some of the other laboratories were out of line (*e.g.*, 16, 25, and 11) inquiry did not disclose any deviations from the method and therefore there was no justification for rejecting the results. On one procedure—the Sanders-Sager butyl alcohol photometric reading—there was very limited and incomplete data so very little analysis was possible.

(a) *Overlapping.*—All of the data, divided according to laboratories for each method, were plotted on charts as shown on Figures 1a to 1d. Each of these charts indicates the variation in the results from laboratory to laboratory, between analysts and between samples at each level of percentage of raw milk used. The right side of Figure 1a shows the data on the Sanders-Sager method with the aqueous photometric reading. This type of chart was made for each of eight procedures involved in the collaborative experiment. From the right side of Figure 1a it can be seen that:

(1) Some laboratories obtained positive results on a sample containing 0% raw milk.

(2) No laboratory obtained a zero result for 0.1% raw milk.

(3) The variation in the results increased with increasing amounts of raw milk present in the samples.

(4) Some laboratories, such as laboratory 16, had relatively high results on *all* levels of percentage of raw milk, while others, such as laboratory 20, had relatively low results on *all* levels.

(5) The variation between samples within any one laboratory was very small, thus showing that the variation between laboratories was not due to differences between samples as they were received by collaborators.

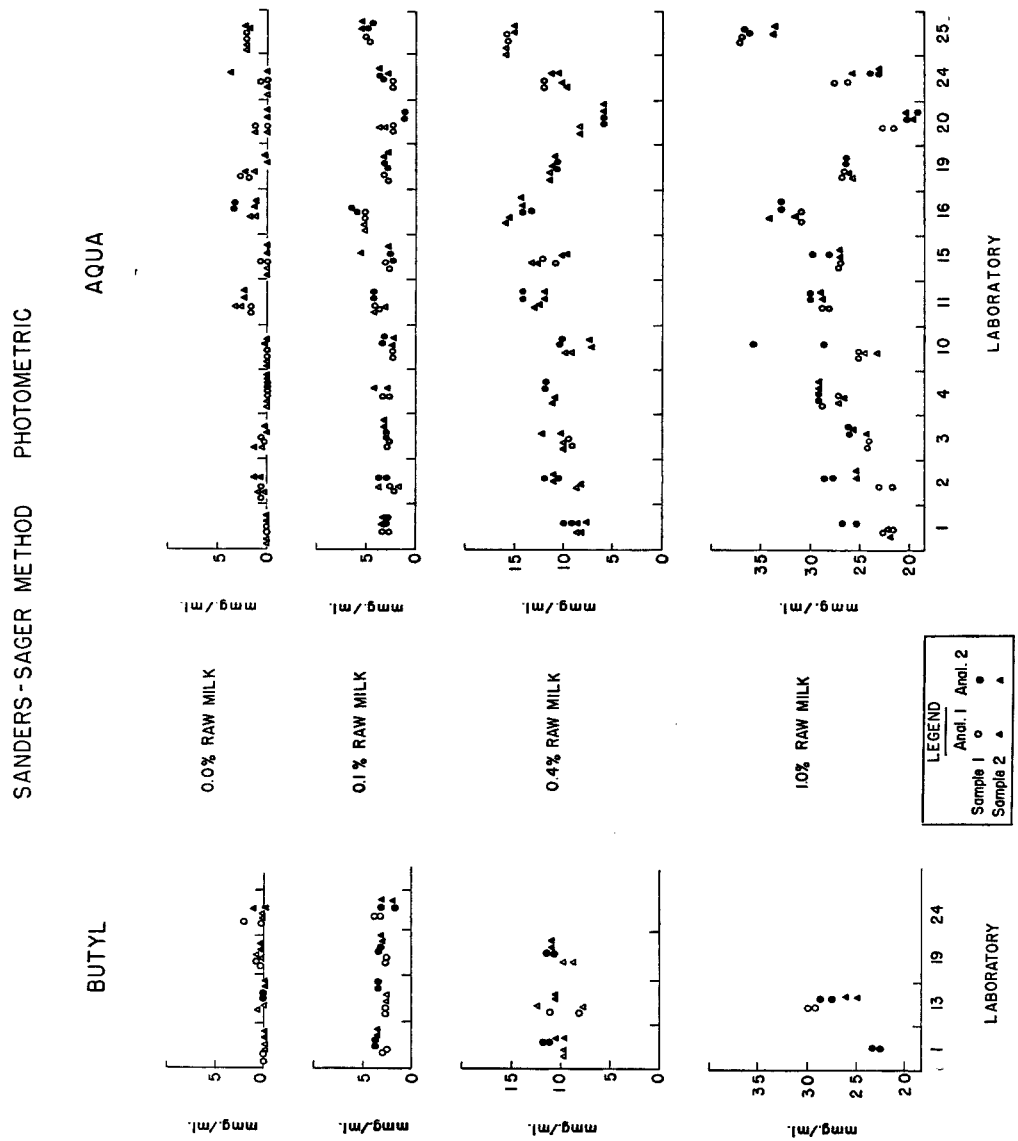

FIG. 1a.—Individual observations according to laboratory, by the Sanders-Sager Method

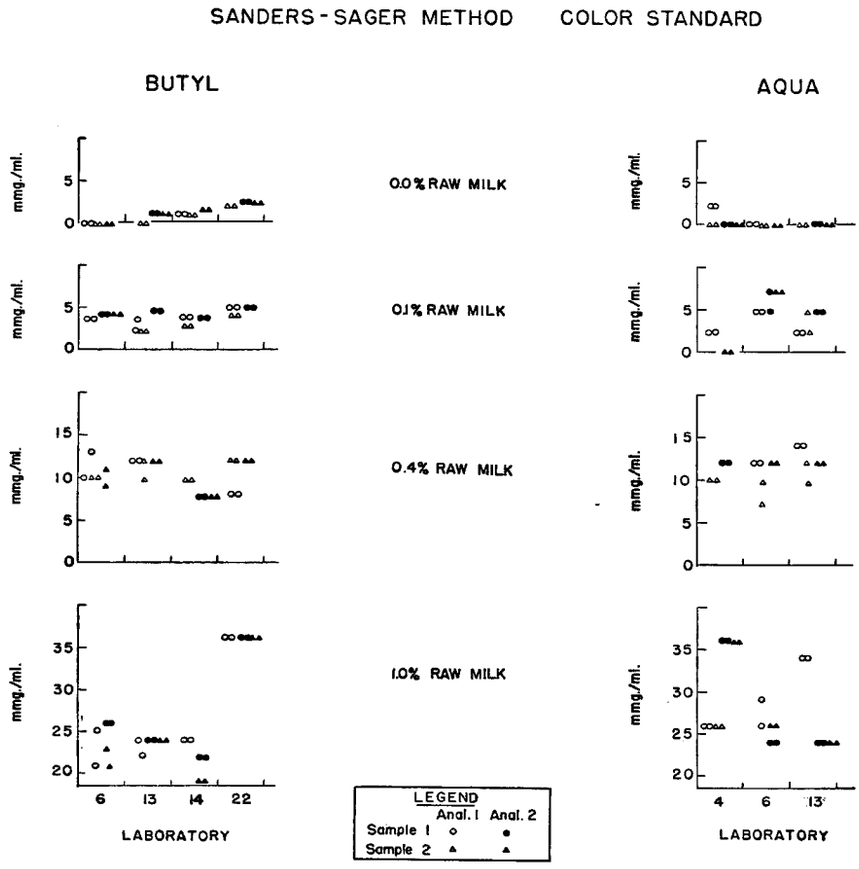

FIG. 1b.—Individual observations according to laboratory, by the Sanders-Sager Method using the Color Standards Measurements on the Butyl and Aqueous Phases.

(6) The differences between analysts within one laboratory was also very small compared with the differences between laboratories.

(7) In only four laboratories was there overlapping of results between the 0% and 0.1% levels of raw milk (laboratories 11, 19, 20, and 24). There was no overlapping between results on the levels 0.1% and 0.4% raw milk.

This type of comparison was made for each of the seven procedures. The one procedure for which there was insufficient data for comparison with other methods (the Sanders-Sager butyl alcohol photometric reading) had no overlapping between results on any of the levels. The methods were ranked in order on the amount of overlapping present, with the

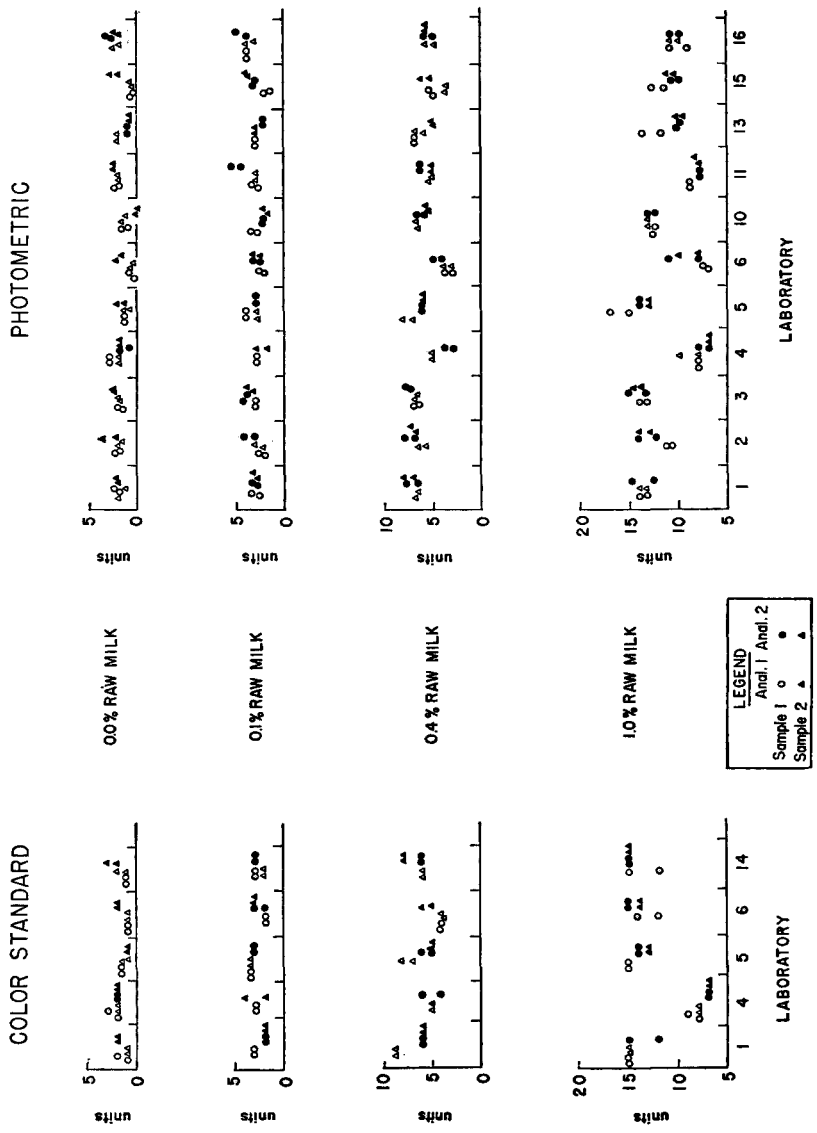

FIG. 1c.—Individual observations according to laboratory, by the Gilcreas-Davis (Long Method) using the Color Standards and Photometric Determinations.

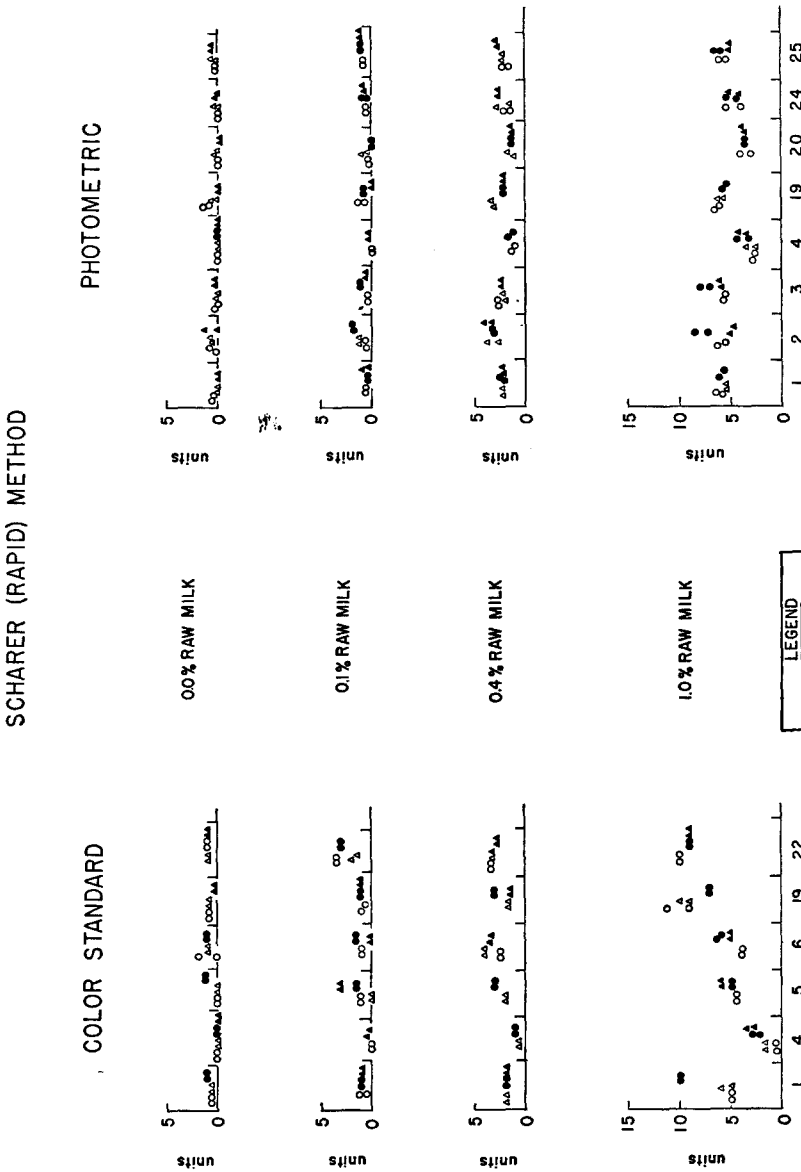

FIG. 1d.—Individual observations according to laboratory, by the Scharer (Rapid) Method using the Color Standards and Photometric Determinations.

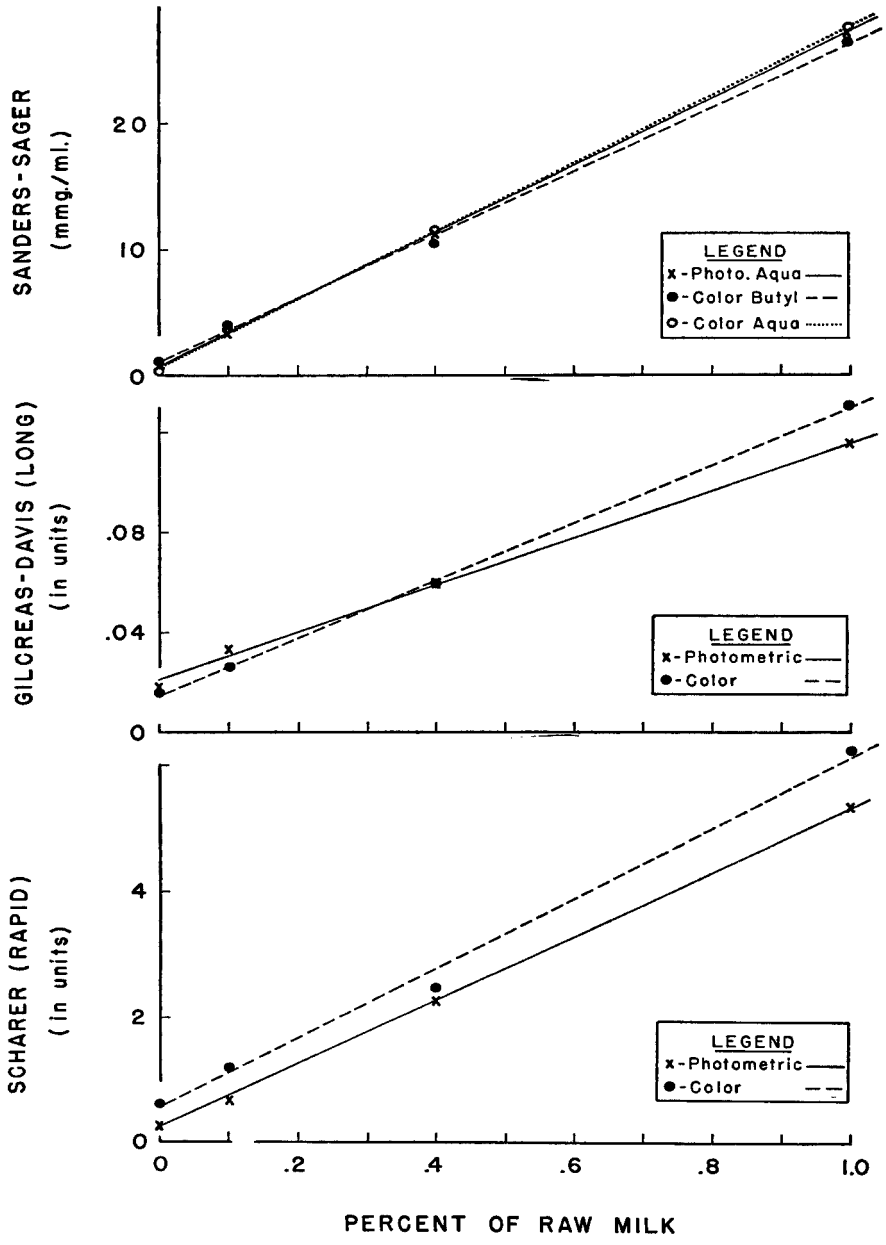

FIG. 2.—Linear Relationship between the average determinations by each method and the actual percent of raw milk in the samples; also the Specificity (Average Determinations at Zero Percent Raw Milk).

best method given a rank of 1 and the worst a rank of 7. (A table of these ranks will be shown later for the four criteria used in comparing the methods.)

(b) *Linearity.*—The average results from all laboratories, all collaborators, and all samples were obtained for each method and each level of raw milk percentage. These average results were plotted against the per cent raw milk present in the sample to investigate the linearity of this relationship. Figure 2 shows the averages and the straight lines, fitted to the plotted points. It can be seen that for the Sanders-Sager aqueous photometric reading, the plotted points fall directly on the straight line. For other procedures, such as the Gilcreas-Davis photometric reading, the observations deviated considerably from the fitted straight line. The seven procedures were ranked according to linearity with the method whose averages fall directly on a straight line given the rank of 1. (A statistical Chi-square test for "goodness of fit" was also calculated to verify the conclusions obtained from observation of Figure 2.)

(c) *Specificity.*—From Figure 2 can be observed which method has the average result on 0% raw milk that most closely approaches zero, *i.e.*, the Sanders-Sager comparison with color-standards in the aqueous solution. This method was given a rank of 1 and the other procedures ranked in order on this criterion from 1 to 7. This observational ranking was checked arithmetically by converting the average results for the 0% raw milk level with the other six procedures to results equivalent to the Sanders-Sager method.

(d) *Reproducibility.*—By means of statistical analysis of variance and variance components* the reproducibility of the seven procedures were compared. Reproducibility in this case means how closely the results obtained by one analyst can be checked by another analyst at another laboratory. (This is derived from the sum of variance components for analysts, samples, laboratories, and random sampling variation.†)

In order to compare methods directly the standard error for reproducibility of each method must be divided by the average phosphatase value for that method, since the results from the various procedures are measured in different units. One hundred times this quotient gives what is ordinarily called the coefficient of variation, sometimes abbreviated to C.V. Figure 3 shows the reproducibility of the various methods in terms of the coefficient of variation (C.V.) for the four levels of per cent of raw milk and also for an average of the three upper levels (0.1%, 0.4%,

* The methods of computation will not be given here, but references to typical computational procedures and the statistical logic behind them can be found in: "Variance Components" by S. Lee Crump, *Biometrics*, 2, 7 (1946); *Statistical Methods in Industry*, L. H. C. Tippett, 1943, Iron and Steel Industrial Research Council of the British Iron and Steel Foundation; and *The Estimation of Components of Variance*, H. E. Daniels Supplement to the *J. Royal Stat. Soc.*, 6, No. 2, 186–197 (1939). Some phases of the analysis are covered in "Statistical Methods" by George W. Snedecor, 4th Ed., Iowa State College Press (1946), pages 278 and 289.

† If σ_A^2, σ_L^2, σ_S^2, and σ_E^2 are the variance components for analysts, laboratories, samples, and random sampling variation, respectively, then, the standard deviation for reproducibility $= \sqrt{\sigma_A^2+\sigma_L^2+\sigma_S^2+\sigma_E^2}$.

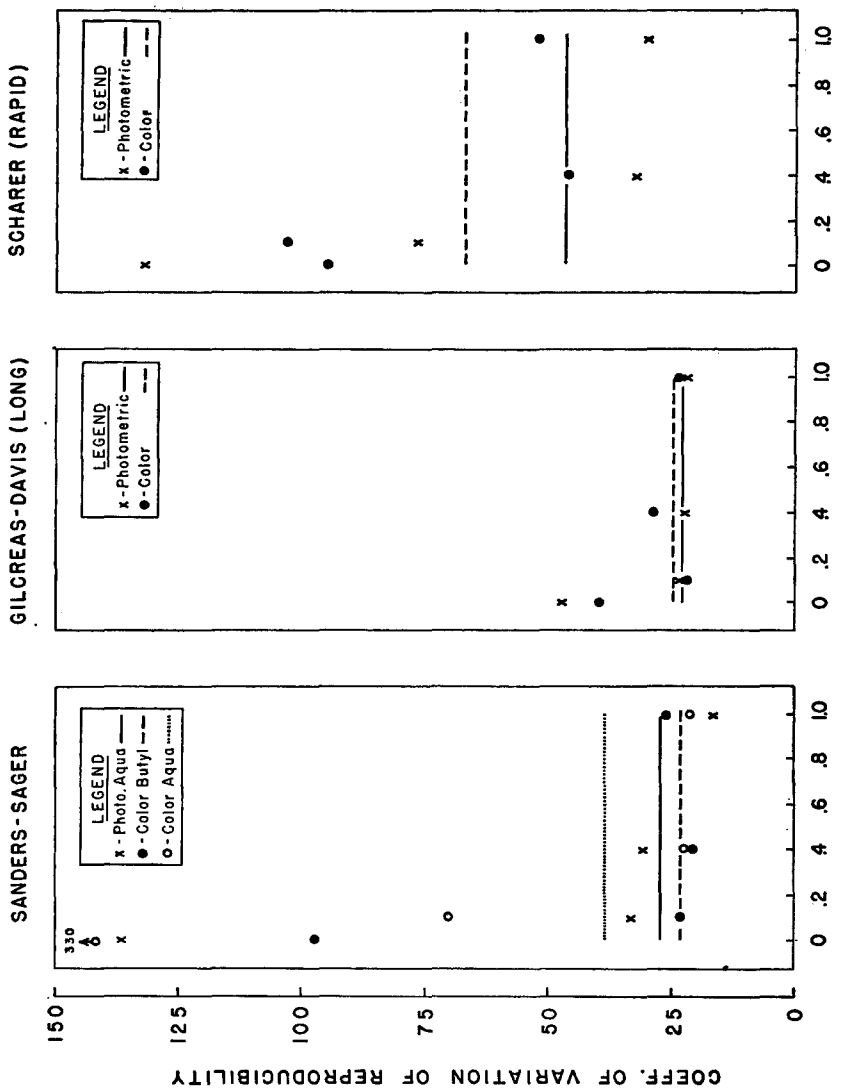

FIG. 3.—Comparative Reproducibility of the Various Methods in Terms of the Coefficients of Variation for Reproducibility (Lines are averages of the three upper levels of per cent raw milk).

and 1.0%). This latter average, instead of the average of all four levels, is used because for the 0% level the closer to zero its average is, the larger the C.V., and because specificity (or closeness to zero of the average of this level) is rated separately.

The seven methods were ranked on this criterion of reproducibility, giving the procedure with the smallest average C.V. the rank of 1 (the Gilcreas-Davis photometric procedure) and the procedure with the largest average C.V. the rank of 7 (the Scharer color standards procedure).

TABLE 2.—*Ranks of various methods on the four criteria*

	NO. OF LABS.	REPRODUCIBILITY (d)	LACK OF OVERLAPPING (a)	SPECIFICITY (c)	LINEARITY (b)	TOTAL
Sanders-Sager Phot. Aq.	12	4 (.270)*	2	2 (0.8)†	1	9
Sanders-Sager Col. Butyl	4	2 (.232)	1	4 (1.1)	4	11
Sanders-Sager Col. Aq.	3	5 (.382)	4	1 (0.3)	6	16
Scharer Phot.	11	6 (.467)	3	3 (1.3)	5	17
Scharer Color	5	7 (.673)	6	5 (2.5)	7	25
Gilcreas-Davis Phot.	8	1 (.230)	7	7 (3.5)	3	18
Gilcreas-Davis Color	6	3 (.249)	5	6 (3.0)	2	16

* The figures in parenthesis in the column for "Reproducibility" are the actual values of the average coefficients of variation. Thus it can be seen that there is little difference in actual values between the ranks 1, 2, 3, and 4.

† The figures in parenthesis in the column for "Specificity" are the actual grand averages for the 0% unpasteurized milk level converted to the equivalent of Sanders-Sager Photometric Aqueous units to make them directly comparable.

(e) *Combining All Criteria.*—In considering all criteria together a table of ranks was set up as in Table 2. Reproducibility (d) was considered most important and linearity was considered least important. In the last column of the table the ranks are added without weighting. From the last column of Table 2 it appears that the Sanders-Sager Photometric Aqueous procedure is the best method when each is given equal weight. The same order of groups of methods (Sanders-Sager, Gilcreas-Davis, Scharer) is retained when the factors are weighted in the order (d), (a), (c), (b).

SUGGESTED CHANGES IN THE SANDERS-SAGER METHOD

The Sanders-Sager method as submitted to the collaborators was identical to that previously published[1] except that it was abstracted so that it referred only to milk.‡

The experience with the method in this study indicates that the following minor changes are desirable for increased accuracy, precision, and clarity.

‡ To conserve space the method is not repeated here, but it will be published in the Seventh Edition of the *Book of Methods*. The method will appear in detail for milk, and directions for use with other dairy products will incorporate only those instructions required for use with the particular product, with cross references to the milk sections for common operations.

(1) *Incubation by samples* (not by groups).—After the data had been tabulated it was noted that some laboratories reported results which were consistently higher than the others and an inquiry was made to determine the technique used in handling the milk-buffer substrate mixtures. It was found that some laboratories incubated the mixtures tube by tube, while others added the substrate to all the milk samples before incubation as a group, allowing the first tube to stand at room temperature for as long as 15 minutes. The present directions were written for carrying out a single determination and they should be modified by a supplementary statement to indicate that the individual tubes should be incubated immediately after addition of the substrate, for exactly one hour. Since initial hydrolysis of the substrate proceeds at an appreciable rate at room temperature, it is believed that the incorporation of such a statement will lead to more consistent results among laboratories. It should be noted, however, that such a procedure will not affect an overnight incubation method, such as the Gilcreas-Davis, because the hydrolysis apparently proceeds to completion during this period and is little influenced by small variations in time and temperature.

(2) *Stability limits to color standards.*—The present directions do not indicate the stability limits of the color standards and there is little information in the literature on this point. Some work by Sanders[3], indicates that the color standards are stable for only one to two weeks and then only if stored in the refrigerator. One collaborator, however, reported that his standards were off color after standing overnight in the refrigerator. Some effort should be made to develop artificial inorganic color standards for use with this method if there is sufficient demand for this type of measurement. In the meantime the method should be modified by a statement that the standards should be prepared fresh weekly.

(3) *p*H *ranges.*—Several collaborators inquired as to the significance of the *p*H values incorporated in the method, and what should be done if the values actually found differ from those stated. A statement should be added at the end of the method to the effect that *p*H variations of 0.15 unit or less are not significant but if larger variations are encountered at the incubation or color development steps the *p*H should be adjusted by drop-wise addition of *N* sodium carbonate or *N* hydrochloric acid. Such variations are rarely encountered with milk and only infrequently with other dairy products. When dealing with a single product, *p*H values need to be checked infrequently, as when new buffers are prepared.

In the case of those cheeses where there is no information regarding age, the average buffer in the cheese group will usually be satisfactory. The *p*H may also be checked and adjusted to the optimum if necessary.

In connection with the standard borax buffer, a typographical error was incorporated into the original Bureau of Standards certificate, which

[3] Sanders, George P., *personal communication.*

has since been corrected. The correct weight of borax for a 0.01 *M* solution is 0.9536 g diluted to 250 ml.

(4) *Reagent.*—Some laboratories have encountered difficulty in obtaining and maintaining a BQC reagent of suitable quality and these may obtain unsatisfactory results. The Associate Referee has maintained a single lot of BQC (Applied Research Institute) for over four years without deterioration, as judged by the reproducibility of the standard curve. Subsequent lots from this source have also proved satisfactory. The description of the reagent in the method should be supplemented by a statement that new lots of the reagent should be tested by checking the standard curve and that this procedure should be repeated semiannually. The Associate Referee maintains the parent lot of BQC in the original tightly stoppered bottle stored in a refrigerator, removing 100 mg portions to a weighing bottle (also stored in the refrigerator) to be used for the preparation of the solution as required. Whenever the bottles are removed from the refrigerator they are allowed to come to room temperature before being opened, in order to avoid moisture condensation on the reagent. Storage of this reagent, as well as disodium phenyl phosphate, in a desiccator at room temperature is also satisfactory.

A purification procedure for the disodium phenyl phosphate involving a butyl alcohol extraction of developed indophenol should also be inserted.

(5) *Other minor changes.*—The directions have been edited to conform to the style of the *Book of Methods* and some changes of an editorial nature have been made. The phrase "in a vessel of cold water" has been added after "cool to room temperature" (in the heat inactivation step) to indicate that the cooling should be performed rapidly to avoid unnecessary standing at higher temperatures.

(6) *Status of methods as applied to various dairy products.*—Collaborative work has been performed with this method for fluid milk, cheddar type cheese, and soft uncured cheeses, and since the methods as applied to these products are now first action, they should be raised to official status. The method is now tentative for other dairy products except cream (for which it is first action) and it should be raised to official for these products, since the fundamental principles are the same regardless of product, and since no deficiencies as applied to other dairy products have been brought to the attention of the Associate Referee. Some preliminary experiments performed by the Associate Referee with ice cream mix confirms the applicability of the Sanders-Sager method to this product.

THE GILCREAS-DAVIS (LONG) PROCEDURE

The Gilcreas-Davis method made an unexpectedly strong showing in reproducibility and linearity in this collaborative study. In fact, the photometric modification showed the lowest coefficient of variation of the seven tests. Discussions with laboratory directors of Public Health

organizations have revealed the necessity for a method which is capable of handling many samples simultaneously for the routine control of milk. Such a method requires operations of a type in which the time factors need not be controlled closely and which therefore are capable of serial operations. Further, the method should be simple, foolproof, and easily interpreted, so that it can be performed routinely by technicians. Although the Gilcreas-Davis test ranks relatively low in lack of overlapping at any one laboratory (criterion *a*) and has a relatively high blank (criterion *c*) it is very reproducible (criterion *d*). The overnight incubation, and the fact that the final reaction product is stable and can therefore be developed at any time and allowed to stand until measured, give this procedure an advantage over the Scharer (Rapid) method for the routine control of fluid milk.

Some collaborators, including the Associate Referee, found that the standard curves and blanks by this method were not reproducible. Some collaborators obtained straight line photometric standard curves while others did not. One collaborator reported that the curves were extremely variable with blank readings (in optical density) varying from 0.14 to 0.37. In view of the results, however, apparently these objections are not too serious. Some of the other objections to this method, such as the low buffer capacity of the buffer substrate and the fact that the color development reagent is nonspecific, are unimportant if the procedure is confined to the examination of milk and cream only.

In view of the necessity for a multiple-sample type of test for the routine control of milk, and since the Gilcreas-Davis method most nearly conforms to the requirements (see also the discussion of the Rapid test below), it is recommended that this procedure be reinstated as first action, and that it be supplemented further with directions for reading the color photometrically as was done in this study. The directions as sent out were confusing to one set of collaborators and will therefore be edited for clarity.

Details of the method will be published in the 7th ed., *Methods of Analysis*, 1950.

THE SCHARER (RAPID) PROCEDURE

The Scharer (Rapid) test, especially when evaluated with the color standards, ranked the lowest of all the methods when judged by the four previously described criteria. This is not surprising since the previous study by the Associate Referee[4] showed that the incubation, color development, and protein precipitation conditions were not optimal. Several collaborators noted that two drops of BQC reagent were inadequate for more than 10 micrograms of phenol in the final aliquot, and that 15 minutes were insufficient for complete color development. In two

[4] *This Journal*, 29, 129 (1946).

cases turbid filtrates were obtained which did not clear up on addition of pyrophosphate. It is significant perhaps, that of those laboratories which indicated a preference of methods, eight preferred the Sanders-Sager procedure, two the Gilcreas-Davis procedure, and none the Scharer test used in this study.

It must be acknowledged, however, that this Scharer procedure is the prototype of all phosphatase tests using the BQC reagent, and that the Sanders-Sager method is actually a modification of the Scharer procedure.

SUMMARY

A statistically designed collaborative study has been performed involving the Sanders-Sager, the Gilcreas-Davis (Long Test), and the Scharer (Rapid Test) phosphatase methods. The statistical analysis of 1,176 individual determinations from 20 laboratories on eight samples (four of which were internal duplicates) at the 0%, 0.1%, 0.4%, 1.0% raw whole milk levels indicated that the Sanders-Sager procedure with the final color measured photometrically in aqueous solution was the best procedure, as evaluated by a combination of four criteria as herein set forth (p. 529), for each of the seven methods.

The Gilcreas-Davis method ranked next to the Sanders-Sager method, and since its operations are particularly amenable to serial manipulations it is recommended for the routine control of pasteurization of fluid milk and cream.

The Scharer (Rapid) method is not outstanding in any respect and ranked last in the composite rating.

ACKNOWLEDGMENT

The splendid cooperation of the following laboratories and collaborators who participated in this study is gratefully acknowledged.

Barbara Lang, Catherine Glynn, of the Department of Health, State of Connecticut
J. E. O'Brien, Eleanor Weeber, Department of Health, State of New York
Frank V. Kosikowsky, Catherine V. Work, Cornell University, N. Y.
Jane M. Flynn, Field Sanitation Laboratory, Buffalo, N. Y.
E. Kaplan, Irmgard Wolf, Baltimore City Health Dept., Md.
L. H. Burgwald, T. V. Armstrong, Ohio State University
Samuel A. Lear, H. G. Foster, Rutgers University, New Brunswick, N. J.
Robert C. Thomas, Harold L. Faig, U. S. Public Health Service, Cincinnati, O.
Ellen D. McKernan, R. M. Radford, National Dairy Research Laboratories, Oakdale, Long Island
and the following in the Food and Drug Administration:
L. C. Mitchell, William Horwitz, Minneapolis, Minn.
John H. Bornmann, Leon E. Wener, Chicago, Ill.
George McClellan, John F. Weeks, Jr., New Orleans, La.
Curtis R. Joiner, Matthew L. Dow, St. Louis, Mo.
H. I. Macomber, S. M. Walden, Baltimore, Md.
Mildred L. Rollins, Denver, Colo.

Sam D. Fine, F. J. McNall, Cincinnati, O.
C. G. Cunningham, John F. Mallon, Boston, Mass.
Wm. H. Munday, Harry W. Conroy, Kansas City, Mo.
L. W. Ferris, H. P. Eiduson, Buffalo, N. Y.
J. W. Cook, H. J. Meuron, San Francisco, Calif.

RECOMMENDATIONS*

It is recommended—

(1) That the Sanders-Sager method for the detection of residual phosphatase be adopted as official for milk and cream, cheddar type cheese, and soft uncured cheeses (cottage, cream, etc.), and as first action for all other dairy products.

(2) That the minor clarifying changes in the Sanders-Sager method recommended in this report be adopted.

(3) That the Long Test (Gilcreas-Davis) be adopted as first action, made applicable only to fluid milk and cream, together with an alternate photometric procedure.

(4) That the Rapid test for phosphatase, 22.49–22.57, be dropped.

(5) That the work on the phosphatase test be continued.

REPORT ON METHODS FOR PREPARATION OF BUTTER SAMPLES

By ALBERT L. WEBER (U.S. Food and Drug Administration, Federal Security Agency, New York, N. Y.), *Associate Referee*

The following work was performed in line with the recommendation of Committee C: "That the use of mechanical stirring devices and of artificial methods of cooling, suitable for use in the present official method for preparation of butter samples, be further studied.

Three lots of salted butter from different localities and one of sweet butter were obtained. The butter was prepared for *moisture analysis* by five different methods. The methods are:

I. The official hand shaking method, A.O.A.C. par. 22.108.
II. The butter melted at temperatures greater than 39°C; hand shaken under running water at 22°C.
III. The Meuron Method (*This Journal*, **29**, 126 (1946)
IV. Softening the butter by the official method, but shaking in a mechanical bottle shaker at moderate speed.
V. Melted butter at temperature greater than 39°C,. shaking like Method IV, but with a fan blowing on shaker.

The containers used were regular Mason jars fitted with a flat metal disc with rubber gasket cover secured by a threaded ring.

The butter was softened (except in Methods II and V) according to

* For report of Subcommittee C and action of The Association, see *This Journal*, 33, 47 (1950).

Vorhes' (*This Journal*, **29**, 121) suggested redraft of paragraph **22.108**, *Methods of Analysis*. Samples were collected in a manner similar to that of Vorhes (*This Journal* **29**, page 123).

The butters used were as follows:

(a) Salted butter from Minnesota, described as a "dry butter."
(b) Salted butter from Iowa, described as a "wet butter."
(c) Salted butter from New York State.
(d) Sweet butter from Iowa.

The room temperatures varied between 28°C. and 32.5°C. Because of these high room temperatures, great care had to be taken that the butter did not become too soft before shaking was started.

Results of the above work are presented in the accompanying table.

TABLE 1.—*Initial results in study of methods for preparation of butter*

BUTTER	METHOD				
	I OFFICIAL	III MEURON	IV SHAKER AFTER SOFTENING	II MELTED, SHOOK UNDER WATER AT 22°	V SHAKER AFTER MELTING
	per cent moisture				
a-1	16.52	16.58	16.53		
	16.60	16.62	16.52		
a-2 Top	16.52	16.58	16.46	16.68[1]	16.61[1]
Bottom	16.59	16.60	16.41	16.79[2]	16.59[2]
b-1	16.63	16.67	16.63		
	16.63	16.68	16.69		
b-2 Top	16.55	16.53	16.61	16.55[1]	16.56[1]
Bottom	16.57	16.55	16.63	16.57[2]	16.53[2]
c-1	16.19	16.27	16.25		
	16.23	16.22	16.15		
c-2 Top	16.12	16.26	16.18	16.04[1]	16.12[1]
Bottom	16.22	16.24	16.17	16.17[2]	16.04[2]
d-1	17.68	17.75	17.65		
	17.69	17.68	17.70		
d-2 Top	17.66	17.56	17.56	17.67[1]	17.56[1]
Bottom	17.71	17.59	17.65	17.67[2]	17.52[2]

[1] Samples from middle of jar.
[2] Samples from next to glass.

Curd and salt determinations were also made on the above samples. The apparent results coincide with the moisture determinations and therefore are not being reported.

Two subs were used in all methods. On Methods I, III, and IV, duplicate aliquots were used in 1 sub while in the other sub 1 aliquot was taken from the upper third of the jar and 1 aliquot from the bottom third of the jar. On Methods II and V, 1 aliquot was taken from the middle of the jar and 1 aliquot from next to the wall of the jar.

The results show that there is no appreciable difference between any of the methods.

No work was done on the loss of moisture. The work of the previous Associate Referree in his last report (Meuron, H. J., *This Journal*, **31**, 318, 1948), indicates that this was not necessary.

It is recommended*

(1) That collaborative work be performed on the above methods.

(2) That the methods **22.108** and **22.109**, preparation of butter samples, and the tentative method, *This Journal*, **31**, 91 (1948), be made procedures and that the Referee and Associate Referee make diligent effort to devise one or more methods, whether or not involving the use of mechanical shakers, stirrers, etc. that will be satisfactory to analysis.

REPORT ON FAT IN DAIRY PRODUCTS

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

A Subcommittee of the American Dairy Science Association of which the author is chairman[1] initiated as study of the Babcock test for milk in comparison with the Mojonnier method, a mechanized modification of the Roese-Gottlieb method. The results of the study show that by increasing the volume of the sample and eliminating the meniscus on the fat column the Babcock test can be standardized to give results agreeing with those by the Mojonnier method.

The Modified Babcock method is simple, fundamentally sound and accurate, as indicated by the fact that five experienced laboratory technicians obtained excellent agreement with the Mojonnier method on 135 samples of unpreserved milk. The Modified Babcock Method follows:

Heat milk sample to 35°-38°, mix thoroly by pouring from one container to another three to four times. Fill an 18.05 ml pipette so that the upper surface of the milk is at the graduated mark on the draw tube, allow pipette to drain into test bottle for 10–15 seconds, and blow out last milk in the tip. Add 15–17 ml of sulfuric acid (sp. gr. 1.82–1.83 at 20°) and proceed as in the original Babcock Method. Add 2–3 drops of colored mineral oil (glymol sp gr not to exceed 0.85 at 20°) as each bottle is taken from the water bath at 57.3 to 60°. The oil must not drop into the fat, but flow down the sides of the neck. Measure the fat from the bottom of the column to the fat-glymol line.

The adoption of the Modified Babcock Method is recommended.*

[1] Other members of the committee are L. H. Burgwald, B. L. Herrington, and E. L. Jack of the Ohio, New York, and California Agricultural Experiment Stations, respectively.

* For report of Subcommittee C and action of The Association, see *This Journal*, **33**, 48 (1950).

REPORT ON FROZEN DESSERTS

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

The Associate Referee on Frozen Desserts spent most of his time in a review of the literature on his subject, in trying to establish contacts and get his feet on the ground.

No specific program was recommended by Committee C.

A review of *Methods of Analysis*, 6th Ed., reveals that the only official method for fat (22.149) in ice cream is the Roese-Gottlieb method. Actually some States and municipalities use a modified Babcock Method for fat (Gottlieb, Nebraska, Minnesota, etc.) not only as a sorting method, but as a basis for prosecution. On the other hand, the large dairies in this locality use the Mojonnier method for fat and their control chemists have no faith in any modified Babcock method. Babcock methods have been thoroughly investigated by previous Associate Referees (Horvet, 1915–17; Frary, 1933–34) with results that caused recommendations that the study of these methods be dropped. A study of Babcock methods, therefore, seems futile.

The methods employed for solids and fat in ice cream by the large manufacturers in this vicinity involve the use of the so-called Mojonnier machine. These are essentially the official methods modified as to times and temperatures of drying with the use of vacuum.

The method for overrun in ice cream (**22.144–45**) weight per unit volume of packaged ice cream) was discussed with enforcement officials and the trade, but no work on this phase of the problem has been done by enforcement officials in this vicinity, and the manufacturers check their overrun by weighing a large number of packages and assuming the purported volume of the containers.

A brief review of Hart's original article, *This Journal*, **28**, 600 (1945), indicates that enough collaborative work has been done to make this method official in view of its sound scientific basis.

RECOMMENDATIONS*

It is recommended—

(1) That **22.145**, a method for determining the weight per unit volume of packaged ice cream, be made first action.

(2) That **22.149**, a method for fat in ice cream, be made to conform to **22.25** by substituting the words "Mojonnier fat extraction flask or Rohrig tube" for "Rohrig tube" in line 2 of this paragraph.

(3) That **22.146** (**b**) preparation of sample, last line, be modified to read "(Many satisfactory mixers such as the Waring Blendor are on the market)."

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* For report of Subcommittee C and action of The Association, see *This Journal*, **33**, 48 (1950).

(4) That the effect of modifications introduced in the official methods for fat and solids be studied.

A contributed paper entitled "A New Method for Reconstituted Milk," by G. F. Edwards, is published on p. 855.

REPORT ON EXTRANEOUS MATERIALS IN FOODS AND DRUGS

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

Consistent with the current plans of the Association, each Associate Referee was asked to review the methods assigned to him in preparation for the reclassification of methods and publication of Ed. VII of *Methods of Analysis*. In carrying out this program the various Associate Referees freely consulted together, with the General Referee, and, at the same time, were constantly guided by the Report of Committee on Classification of Methods (*This Journal*, **31**, 63, 1948) which set up four broad groups into which the present "Tentative" methods may be placed. (1) Those which could and should be made official with or without further collaborative study. (2) Those which have been thought, erroneously, to be, from their very nature, incapable of collaborative study. (3) Those which are for the purpose of making rough approximations. (4) Those which are clearly obsolete. Concerning group 2, into which most of the Chapter 42 methods fall, the committee report states: "We believe that any method . . . that is of proven usefulness is, almost by definition, capable of collaborative study. Take as an illustration the methods for the isolation of filth . . . these . . . could and should be studied collaboratively, not as methods of measurement, which they are not, but rather as to whether they do or do not clearly isolate the filth from the food itself. In such a study the collaborator would report a method as 'satisfactory' if no food remained with the filth to obscure it. Satisfactory methods of isolation should then be adopted as official."

It was thus clear that collaboration on the directions for isolation need not extend to the identification of filth elements so isolated. In fact, it is the experience of the Referee and Associate Referees that proficient microscopic examinations for filth can only be made after personal training and discussions with others already thoroughly qualified in this work. In line with the later Report of Committee on Classification of Methods (*This Journal*, **32**, 33 (1949)) which in turn is corroborated by the practical experience of the Referee and Associate Referees, the methods were investigated to determine whether or not they clearly separated filth elements from the food itself.

The adequacy of the mold count procedures has been clearly established by long usage of the tomato and butter methods. When applied to other materials the only phases of the method that require collaborative checking are the preliminary preparations prior to the actual microscopic count.

With these principles as a guide, every method appearing in *Methods of Analysis* 6th Ed., Chapter 42, was reviewed and made the subject of verbal or written collaborative discussions with those analysts doing the great bulk of this work. The recommendations of the Associate Referees are based upon these discussions. Recommendations are made that methods be made official where the published methods will clearly separate filth elements from the food.

The Referee concurs in all of the recommendations of the Associate Referees. Each recommendation of each Associate Referee has been thoroughly studied—in fact to a large extent there was a continuous exchange of opinion between the Associate Referees and the Referee while each report was being prepared—but because of the large number of recommendations being made it appeared advisable not to re-list each recommendation in this report.

REPORT ON EXTRANEOUS MATERIALS IN DRUGS, SPICES, AND MISCELLANEOUS PRODUCTS

By WILLIAM V. EISENBERG (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

Methods **42.8**, **42.9**, and **42.78** thru **42.100** have been subjected to collaborative study. Since the last report of the Associate Referee only one change was made in the above methods. Method **42.83** for ground black and white pepper, was modified to provide for a 25-gram instead of a 10-gram sample. The gasoline extraction is performedby the usual procedure after digesting the decanted pepper in a boiling 0.5% HCl solution. The modified method for **42.83** was included in the collaborative study. The following recommendations* are made.

42.8, 42.9: Delete, since this determination is performed more efficiently by **42.78** and **42.84**. The heading in **42.84** "Whole Marjoram, Savory, and Thyme" should be enlarged to include "Tea, Leafy Crude Drugs, and Condiments."

The methods below are recommended for first action status, the following collaborators having found them satisfactory:

42.78 (including all changes and additions); William V. Eisenberg, Dorothy Scott, William G. Helsel, F. J. McNall, Doris Tilden, Mary Harrigan, Alice C. Goldsworthy.

* For report of Subcommittee C and action of The Association, see *This Journal*, 33, 50 (1950).

42.79, 42.80, 42.81, 42.82: William V. Eisenberg, Dorothy Scott, William G. Helsel, Mary Harrigan, Alice C. Goldsworthy,

42.83, 42.84 (including the addition made after paragraph **42.84**): Flora Y. Mendelsohn, William V. Eisenberg, William G. Helsel, Dorothy Scott, Alice C. Goldsworthy.

42.85: William V. Eisenberg, Albert Wells, F. R. Smith, William G. Helsel, Doris Tilden, J. Armstrong, Robert E. O'Neill.

42.86, 42.87, 42.88, 42.89, 42.90, 42.91, 42.92, 42.93: William V. Eisenberg, Hugh I. Macomber, Shirley I. Walden, William G. Helsel, Frank R. Smith.

42.94: 42.95: Sam Fine, Curtis Joiner, Matthew Dow, William V. Eisenberg. Dorothy Scott.

42.96: William V. Eisenberg, Dorothy Scott, Albert Weber.

42.97, 42.98, 42.99, 42.100: William V. Eisenberg, Robert O'Neill, F. J. McNall, Dorothy Scott, Mary Harrigan, Kenton L. Harris.

The following changes have been made in method **42.83** as submitted for collaborative study: Change **42.83**, line 1, to read as follows: "Weigh 25 g of sample into 400 ml beaker. Add 250 ml of CCl_4. . . . "

Change line 4, beginning with word "transfer" to read "transfer to 2-liter Wildman trap flask **42.1(a)** Add 500 ml of H_2O+10 ml HCl. Boil gently for 15–20 min., cool to room temp., add 35 ml gasoline, and mix thoroly. Fill flask with H_2O . . . etc."

The methods for Canned Fish, Chicken Giblet Paste, and Meat Scraps (*This Journal*, **30**, 101, 1947) have been subjected to collaborative study. The following collaborators have found these methods satisfactory: William V. Eisenberg, Mary Harringan, Dorothy Scott, William G. Helsel, Kenton L. Harris.

REPORT ON EXTRANEOUS MATERIALS IN NUT PRODUCTS

By MARYVEE G. YAKOWITZ (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

The following methods for extraneous materials in nut products have been subjected to the critical consideration of experienced analysts, and in the absence of any adverse criticism, it is recommended* that they be adopted as first action:

42.22: Shelled nuts—filth.

42.23: Peanut butter—preparation of sample.

42.24: Peanut butter—water-insoluble inorganic residue, and excreta.

42.25: Peanut butter—light filth.

This Journal, **30**, 102, (1947): Coarse peanut butter (rocks and decomposed peanuts).

* For report of Subcommittee C and action of The Association, see *This Journal*, **33**, 50 (1950).

The collaborators with whom the methods were discussed were: Robert E. O'Neill, Atlanta; R. T. Elliott, Seattle; Helen T. Hyde, San Francisco; A. H. Wells, Los Angeles; Dorothy B. Scott, New York.

Although no collaborative information is available on the method for shredded coconut (*This Journal*, **30**, 102, 1947), this is the only available method for this material and it is recommended* that it be included as a suggested procedure.

REPORT ON EXTRANEOUS MATERIALS IN BAKED PRODUCTS, CEREALS, AND CONFECTIONERY

By J. F. NICHOLSON (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

In accordance with the report of the Referee on extraneous materials the methods discussed in this report have been submitted for collaborative study. The collaborators who made a study of the methods were: Robert O'Neill, Atlanta District; Joyce Holberg, Minneapolis District; J. Breit, Cincinnati District; Virginia W. Leland and Shirley M. Walden, Baltimore District; J. Phyllis Skyrme, Boston District, all of the U. S. Food and Drug Administration.

RECOMMENDATIONS*

The collaborative opinion was obtained on methods which were published in *This Journal*, **32**, 322, 323, and 324 (1949). In view of the favorable opinions expressed by the collaborators, it is recommended that the present sections **42.26**, **42.27**, **42.28**, **42.29**, **42.30** be deleted and that the above referenced methods be used in place of them, and made first action. In this regard it should be noted that in **32** (p. 322), "(1) Sieving . . . ", and (p. 323) "(2) Direct trapping . . . " applies to both baked goods and alimentary paste, so that the present separate method, **42.37**, should be deleted. Also, the "Insect Eggs in Flour" method should remain a part of the flour procedure and it is recommended for adoption as first action.

On the basis of the collaborative results, **42.31**, **42.33**, **42.34**, **42.35** and **42.36** are recommended for adoption, first action. It is also recommended that **42.38** as amended be adopted, first action.

Since **42.38(a)** and (d) are macroscopic tests it is felt that they are unsuited for continued inclusion in *Methods of Analysis* and should be deleted.

42.38(b) has proved to be unsuited to the handling of some brewer's grits. It is recommended that this section be delected and that the present corn meal method, **42.33**, be substituted for it. Inasmuch as this substitution has been used on a wide variety of brewer's grits and is the only method presently available, it is recommended that it be included as a

* For report of Subcommittee C and action of The Association, see *This Journal*, **33**, 50 (1950).

suggested procedure and submitted for collaborative sudy at an early date.

It is recommended that 42.38(c) and also the method for chewing gum (*This Journal*, 30, 102) be given further collaborative study but left in as the only available procedure.

It is recommended that the pop corn methods (*Ibid.* 32, 116) be included as a suggested method for use in the examination of popped and unpopped popcorn.

The collaborative study of the candy methods was made not on the 42.54 procedures, but on those methods given in *This Journal*, 32, 321–22. Favorable opinions were expressed by all collaborators concerning these methods. It is recommended that 42.54 be deleted and these new procedures be adopted as first action.

42.55 and 42.56 are recommended for adoption as first action.

REPORT ON SEDIMENT TESTS IN MILK AND CREAM

By CURTIS R. JOINER (Food and Drug Administration, Federal Security Agency, St. Louis, Mo.), *Associate Referee*

In a report last year (1), the Associate Referee recommended that the proposed method for the preparation of standard sediment pads, which was a modification of the tentative method (2), be subjected to collaborative study, and that some work be done on the use of standard pads protected by a coating of a colorless transparent plastic cement. This work has been done, and, in addition, a collaborative study of the grading of pads as directed in this present tentative method was carried out.

COLLABORATIVE STUDY OF GRADING OF PADS

For this purpose thirty-six pads were prepared in the laboratory, using known weights of sediment ranging from 0.2 mg. to 14.0 mg. Twelve of the thirty-six pads were made from each of three different sediment mixtures. These mixtures were made with soils which varied widely in appearance. A light-colored soil was used in Mixture I, a fairly dark soil in Mixture II, and a red soil in Mixture III. The same manure sample was used in all three mixtures.

Fourteen discs were selected as representative of a large group of pads which had been obtained in the field testing of milk for sediment. The fifty pads were mounted on cardboard sheets and all were treated with a solution of a colorless, plastic cement. As a result of this treatment none of the pads were altered in appearance during several shipments and numerous handlings.

The collaborators were asked to grade the pads entirely independently, first with a photographic standard strip which has been in use in the Food and Drug Administration for several years, and then after a lapse of two or three days to regrade them using a set of standard pads which had been

TABLE 1.—*Summary of ratings of pads 1–36*

PAD NO.	WEIGHT OF SEDIMENT USED	THEORETICAL GRADE	NO. OF DEVIATIONS FROM THEORETICAL GRADE			
			PHOTOGRAPHIC STANDARDS		ACTUAL STANDARD PADS	
	mg	*mg*	*above*	*below*	*above*	*below*
1	6.0	6.0	0	4	0	2
2	0.2	0	11	0	6	0
3	14.0	12.0	0	1	0	0
4	4.0	3.0	4	0	1	0
5	3.0	3.0	0	1	0	0
6	8.0	6.0	6	0	9	0
7	1.0	0.5	3	0	0	0
8	12.0	12.0	0	1	0	0
9	6.0	6.0	0	5	0	0
10	10.0	12.0	0	7	0	1
11	5.0	6.0	0	14	0	10
12	0.5	0.5	0	0	0	0
13	4.0	3.0	2	0	1	0
14	8.0	6.0	5	1	4	0
15	5.0	6.0	1	13	0	10
16	1.0	0.5	0	0	0	0
17	6.0	6.0	0	8	0	0
18	14.0	12.0	0	0	0	0
19	0.5	0.5	0	2	0	1
20	6.0	6.0	0	8	0	6
21	0.2	0.0	5	0	6	0
22	8.0	6.0	6	0	12	0
23	12.0	12.0	0	3	0	0
24	4.0	3.0	1	0	1	0
25	3.0	3.0	0	1	0	1
26	10.0	12.0	0	12	0	2
27	0.5	0.5	0	0	1	0
28	12.0	12.0	0	1	0	0
29	6.0	6.0	0	10	0	0
30	1.0	0.5	0	0	0	0
31	14.0	12.0	0	0	0	0
32	5.0	6.0	0	7	0	1
33	3.0	3.0	0	1	0	1
34	6.0	6.0	0	3	0	0
35	0.2	0.0	10	0	8	0
36	10.0	12.0	0	8	0	1
Totals			54	111	49	36
% of Total Possible Deviations (792)			6.8	14.0	6.2	4.5

TABLE 2.—*Summary of each collaborator's ratings of pads 1–36*

COLLABORATOR	NO. OF DEVIATIONS FROM THEORETICAL GRADE							
	PHOTOGRAPHIC STANDARDS				ACTUAL STANDARD PADS			
	ABOVE		BELOW		ABOVE		BELOW	
	No.	*per cent*	*No.*	*per cent*	*No.*	*per cent*	*No.*	*per cent*
1	0	0	4	11.1	0	0	2	5.6
2	0	0	11	30.6	4	11.1	0	0
3	1	2.8	6	16.7	2	5.6	2	5.6
4	6	16.7	2	5.6	1	2.8	2	5.6
5	1	2.8	2	5.6	—	—	—	—
6	0	0	6	16.7	2	5.6	3	8.3
7	7	19.4	0	0	3	8.3	0	0
8	6	16.7	0	0	6	16.7	1	2.8
9	5	13.9	1	2.8	3	8.3	2	5.6
10	4	11.1	2	5.6	3	8.3	2	5.6
11	0	0	7	19.4	0	0	4	11.1
12	2	5.6	13	36.1	1	2.8	2	5.6
13	0	0	6	16.7	2	5.6	2	5.6
14	5	13.9	1	2.8	3	8.3	0	0
15	—	—	—	—	3	8.3	1	2.8
16	2	5.6	1	2.8	3	8.3	1	2.8
17	1	2.8	8	22.2	4	11.1	0	0
18	3	8.3	0	0	4	11.1	0	0
19	2	5.6	5	13.9	1	2.8	3	8.3
20	1	2.8	11	30.6	2	5.6	1	2.8
21	5	13.9	0	0	0	0	1	2.8
22	1	2.8	17	47.2	1	2.8	3	8.3
23	2	5.6	8	22.2	1	2.8	4	11.1
Totals	54		111		49		36	
Max.	7	19.4	17	47.2	6	16.7	4	11.1
Min.	0	0	0	0	0	0	0	0
Ave.	2.5	6.8	5.0	13.9	2.2	6.2	1.6	4.5

treated with plastic cement and mounted between sheets of plastic for protection. A total of twenty-three collaborators from five field districts and two Washington divisions of the Food and Drug Administration graded the pads. Twenty-one of them graded the pads with both sets of standards, one used only the photographic standard, and one only the actual standard pads. Both sets of standards consisted of five pads: 0, 0.5, 3.0, 6.0, and 12.0 milligrams. Only men who had had prior experience in grading sediment discs were asked to participate in this study. The collaborators were asked to do the grading according to the instructions in the present tentative method (2), which states in part, " . . . In using standards, the sediment discs of the sample tested should be graded to the nearest standard sediment disc, whether the actual amount of sediment is above or below the standard. . . ."

TABLE 3.—*Sediment mixture I*

PAD NO.	WEIGHT OF SEDIMENT USED	THEORETICAL GRADE	NO. OF DEVIATIONS FROM THEORETICAL GRADE			
			PHOTOGRAPHIC STANDARDS		ACTUAL STANDARD PADS	
	mg	*mg*	*above*	*below*	*above*	*below*
21	0.2	0	5	0	6	0
27	0.5	0.5	0	0	1	0
16	1.0	0.5	0	0	0	0
33	3.0	3.0	0	1	0	1
4	4.0	3.0	4	0	1	0
15	5.0	6.0	1	13	0	10
9	6.0	6.0	0	5	0	0
34	6.0	6.0	0	3	0	0
22	8.0	6.0	6	0	12	0
10	10.0	12.0	0	7	0	1
28	12.0	12.0	0	1	0	0
3	14.0	12.0	0	1	0	0
Totals			16	31	20	12
% of Total Possible Deviations (264)			6.1	11.7	7.6	4.5

The results obtained by the collaborators on the thirty-six pads prepared with known amounts of sediment are given in Tables 1 through 5. Table 1 gives the actual weight of sediment on each disc, the theoretical grade of each, and the number of deviations obtained by the graders above and below this grade for each pad with both types of standards. Table

TABLE 4.—*Sediment mixture II*

PAD NO.	WEIGHT OF SEDIMENT USED	THEORETICAL GRADE	NO. OF DEVIATIONS FROM THEORETICAL GRADE			
			PHOTOGRAPHIC STANDARDS		ACTUAL STANDARD PADS	
	mg	*mg*	*above*	*below*	*above*	*below*
35	0.2	0	10	0	8	0
12	0.5	0.5	0	0	0	0
30	1.0	0.5	0	0	0	0
5	3.0	3.0	0	1	0	0
24	4.0	3.0	1	0	1	0
11	5.0	6.0	0	14	0	10
17	6.0	6.0	0	8	0	0
29	6.0	6.0	0	10	0	0
6	8.0	6.0	6	0	9	0
36	10.0	12.0	0	8	0	1
23	12.0	12.0	0	3	0	0
18	14.0	12.0	0	0	0	0
Totals			17	44	18	11
% of Total Possible Deviations (264)			6.4	16.7	6.8	4.2

TABLE 5.—*Sediment mixture III*

PAD NO.	WEIGHT OF SEDIMENT USED	THEORETICAL GRADE	NO. OF DEVIATIONS FROM THEORETICAL GRADE			
			PHOTOGRAPHIC STANDARDS		ACTUAL STANDARD PADS	
	mg	*mg*	*above*	*below*	*above*	*below*
2	0.2	0	11	0	6	0
19	0.5	0.5	0	2	0	1
7	1.0	0.5	3	0	0	0
25	3.0	3.0	0	1	0	1
13	4.0	3.0	2	0	1	0
32	5.0	6.0	0	7	0	1
1	6.0	6.0	0	4	0	2
20	6.0	6.0	0	8	0	6
14	8.0	6.0	5	1	4	0
26	10.0	12.0	0	12	0	2
8	12.0	12.0	0	1	0	0
31	14.0	12.0	0	0	0	0
Totals			21	36	11	13
% of Total Possible Deviations (264)			8.0	13.6	4.2	4.9

2 gives the total number of deviations from the theoretical grades for each collaborator. From these two tables it can be seen that 79 per cent of all grades assigned with the photographic standards and 89 per cent with the actual standard pads coincided with the theoretical grades. In no case was any pad rated more than one grade above or below the theoretical grade, and only two pads (numbers 14 and 15) were rated both

TABLE 6.—*Distribution of grades for pads 37–50*

PAD NO.	GRADES ASSIGNED WITH PHOTOGRAPHIC STANDARDS						GRADES ASSIGNED WITH ACTUAL STANDARD PADS						PROBABLE GRADE
	0	0.5	3.0	6.0	12.0	AVERAGE	0	0.5	3.0	6.0	12.0	AVERAGE	
		mg	*mg*	*mg*	*mg*	*mg*		*mg*	*mg*	*mg*	*mg*	*mg*	*mg*
37		19	3			0.8		20	2			0.7	0.5
38			1	4	17	10.5				1	21	11.7	12.0
39		22				0.5	1	20	1			0.6	0.5
40	1	3	15	3		2.9		3	14	4	1	3.6	3.0
41		11	11			1.75		10	12			1.9	3.0
42		3	19			2.7			20	2		3.3	3.0
43			11	11		4.5			7	15		5.1	6.0
44		5	15	2		2.7		6	16			2.3	3.0
45				3	19	11.2				3	19	11.2	12.0
46		1	4	13	4	6.3		1	2	15	4	6.6	6.0
47		3	19			2.7		3	17	2		2.9	3.0
48		5	17			2.4		4	18			2.5	3.0
49			8	14		4.9			8	14		4.9	6.0
50			14	8		4.1			16	6		3.8	3.0

TABLE 7.—*Deviations from probable grades for pads 37–50*

COLLABORATOR	PHOTOGRAPHIC STANDARDS					ACTUAL STANDARD PADS				
	ABOVE	BELOW		NO. OF PROBABLE GRADES		ABOVE		BELOW		NO. OF PROBABLE GRADES
	ONE GRADE	ONE GRADE	TWO GRADES			ONE GRADE	TWO GRADES	ONE GRADE	TWO GRADES	
				No.	*Per Cent*					*No.*	*Per Cent*
1		6		8	57.1			5		9	64.3
2		4		10	71.4	2				12	85.7
3	1	1		12	85.7	1		2		11	78.6
4				14	100.0			1		13	92.9
5	1	2		11	78.6	–		–		—	—
6		5		9	64.3			8		6	42.9
7	2	2		10	71.4			2		12	85.7
8		3		11	78.6			3		11	78.6
9	1	3		10	71.4	1		2		11	78.6
10		1		13	92.9					14	100.0
11		7		7	50.0			7		7	50.0
12		9	1	4	28.6	1		5	1	7	50.0
13		3	1	10	71.4			2		12	85.7
14	1			13	92.9	1				13	92.9
15	–			—	—	1				13	92.9
16	2	1		11	78.6	2				12	85.7
17		5		9	64.3	1		3		10	71.4
18	2	1		11	78.6	1				13	92.9
19		3		11	78.6			4		10	71.4
20	2	3		9	64.3	2				12	85.7
21	4			10	71.4	2	1	3		8	57.1
22	1	1	1	11	78.6	5				9	64.3
23	3			11	78.6	1		1		12	85.7
Ave. No. per Grader	0.91	2.7	0.14	10.2	—	0.95	0.05	2.2	0.05	10.8	—
Ave. % per Grader	6.5	19.3	1.0	—	73.1	6.8	0.3	15.7	0.3	—	76.9

above and below. Tables 3, 4, and 5 are a breakdown of Table 1 into the three groups which were made from different sediment mixtures. There are no significant differences in the total deviations in the three groups of pads.

Tables 6 and 7 are a summary of the results of collaborative grading of pads 37 through 50. These discs were obtained in the field testing of milk for sediment and were selected as being representative of several different types of sediment pads which are commonly obtained in making field sediment tests. Some of these pads are very difficult to grade, particularly those which appear to be covered with fine dust and bear practically no coarse particles. Table 6 gives the distribution of grades for each

pad with both types of standards, the average grades and a "probable" grade for each pad. The former are simply the averages, in milligrams, of all grades assigned by the collaborators, while the latter are the standard pad designations, in milligrams, which are the closest to the averages. Table 7 summarizes the distribution of grades assigned by each collaborator. This table shows that 73 per cent of all grades assigned with the photographic standards and 77 per cent with the actual standard pads were "probable" grades.

COLLABORATIVE STUDY OF THE PREPARATION OF STANDARD PADS

Portions of a standard sediment mixture prepared by the Associate Referee (Mixture II, Table 4) were sent to six collaborators. They were asked to prepare duplicate sets of fifteen pads each (0 to 14.0 mg.) from the mixture, starting with separate two-gram portions each time. They were also asked to prepare one or two similar sets of pads from sediment mixtures made up with soil and manure obtained locally. Each analyst was to mount the pads on cardboard sheets and cover them with a protective coating of diluted duco cement.

The method submitted to collaborators was identical to the one presented last year (1) except that the mesh numbers of the sieves used were all changed to U. S. Standard sieve numbers. This change was desirable in order to insure uniformity of sieves being used in the preparation of sediment mixtures. Of six five-inch "40-mesh" sieves on hand in this laboratory, one was found to be actually 33-mesh, four were 38-mesh, and one was 40-mesh. At the suggestion of one of the collaborators three minor changes in the method were later made and sent to all analysts participating in this work. The method also includes two or three minor changes which were found to be desirable after studying the standard pads submitted by the collaborators.[1]

DISCUSSION OF RESULTS

Pads were prepared and submitted by six different analysts. Four sets of 15 discs each were received from three of them, three sets from two, and two sets from one. All but the last prepared duplicate sets from the mixture made up by the Associate Referee. All of the pads submitted were studied critically. First, each set of pads was examined as a group, then the various sets of pads prepared by an individual collaborator were compared to each other, and finally each pad was compared individually to the corresponding pad prepared by the writer. The five outstanding defects of the collaborative standards are discussed below.

(1) The biggest single defect found in the pads was the uneven distribution of sediment. This defect was present in a majority of the pads submitted by two different collaborators, but it was noted in relatively few of the other standard discs

[1] Details of the method are published in *Methods of Analysis*, 7th Ed., 1950.

studied. However, the pads prepared by these two analysts on which the sediment was evenly distributed were rated as excellent when examined critically. This defect is a very serious one, since pads on which the sediment is not fairly evenly distributed are almost impossible to rate in their proper relationship to other standard discs. Therefore, such pads should not be used as standards for rating unknowns. The method as submitted to the collaborators contained instructions for distributing the sediment evenly over the discs, but these two analysts did not make any comments on this point. After studying the above described pads the following statement was added to the method: "Do not use as a standard any pad on which the sediment is not evenly distributed."

(2) The second most serious defect found was the staining of pads caused by extraction of pigments from the sediment by the solvents in the diluted cement used as a protective coating on the pads. Two sets of discs prepared by one collaborator from his own sediment mixture were stained green to an extent that was objectionable. This analyst stated that the manure he used was from a pasture fed cow. The Associate Referee obtained a portion of this manure and found it impossible to treat it with the plastic cement mixture as directed in the method without extracting objectionable amounts of chlorophyll from it. One other set of pads was stained slightly, but not seriously. None of the discs prepared from the Associate Referee's sediment mixture were stained. As a result of this experience the method was changed to prohibit the use of the protective coating of diluted plastic cement on standard discs if it results in the staining of the pads.

(3) There was some variation in the apparent particle size of sediment prepared by different individuals. That on one set of pads was somewhat larger and that on another appeared to be slightly finer in size than the sediment mixture made up by the Associate Referee. However, these differences were not so great as to cause any marked change in the appearance of the pads. In the absence of any knowledge to the contrary, it must be assumed that all collaborators used U. S. Standard sieves as called for in the method. The other factors which are most likely to cause variations in particle size are the method of grinding and differences in the nature of the materials used. Since it is almost impossible to standardize the latter, the method was changed to require that all materials be ground by hand with a mortar and pestle. This type of grinding should produce less variation in the particle size of material passing through a No. 40 sieve than would grinding with the various types of mills that are on the market.

(4) A few of the pads submitted by one analyst had some brown and some greenish-yellow stains on them. He reported that all of the stained pads had been prepared after his supply of skimmed milk had soured. Pads prepared in an identical manner from the same batch of milk remained normal, while those made from the soured milk became discolored and moldy while they were being air-dried overnight. The collaborator concluded that the sour milk was more difficult to remove from the pads by aspiration. He realized these pads were defective, but because of lack of time he was unable to make them over. Because of this experience the part of the method which called for "skimmed milk" has been changed to "sweet skimmed milk."

(5) The pads submitted by one collaborator had an effective area one inch in diameter instead of the 1⅛ inch specified in the method. The set of pads prepared by this analyst from his own mixture was the darkest set received, and they appeared to conatin somewhat more real fine black sediment than did any of the other pads. This analyst reported that he had used dark soil and "Eponite" in place of charcoal. A sample of the "Eponite," ground to pass through a No. 40 sieve, was compared microscopically to a comparable sample of charcoal. The general type of fracture exhibited by the "Eponite" was not the same as that of the charcoal. This

difference might have accounted for at least part of the difference in appearance of the two sets of pads.

Aside from the specific defects discussed above, the various sets of pads were rated as very good. In all sets there was approximately the proper proportional increase in the amount of sediment on the pads in going up the series. In general, the various duplicate sets appeared to be good duplicates, and with very few exceptions the pads very closely matched those prepared by the Associate Referee.

SUMMARY AND CONCLUSIONS

In the check grading of thirty-six standard pads by twenty-three collaborators, 79 per cent of all grades obtained with the use of photographic standards and 89 per cent with actual standard pads were the same as the theoretical grades for the pads. In the grading of fourteen discs obtained in the field testing of milk, 75 per cent of all grades were identical to the "probable" grades calculated from the averages of ratings assigned by all collaborators. Since the estimation of sediment by comparison with standards is far from being a precision measurement, these results are considered very good.

A number of defects found in standard sediment pads prepared by six different analysts are discussed in detail. Most of these defects pointed up weaknesses in the method which have been corrected. Aside from these specific factors, all of the collaborative standard pads were satisfactory.

RECOMMENDATIONS*

It is recommended—

(1) That the method presented in this report be substituted for the section headed "**B**(**d**) *Preparation of standard sediment discs*" in the method for Sediment in Fluid Milk which was adopted as tentative in 1947(2);

(2) That, in order to keep the directions in the method consistent, the second sentence of the second paragraph of **B**(**e**) "*Checking sediment testers*" beginning, "Thoroly mix 2 gm . . . " be changed to "Place 2.00 g of the oven-dried mixture described in **B**(**d**) in a 100-ml volumetric flask and dilute to volume as directed in **B**(**d**) beginning, 'and moisten with 5 ml. of 1% aerosol . . . '."

(3) That the entire method for Sediment in Fluid Milk (2), amended as recommended above, be adopted as first action.

ACKNOWLEDGMENT

The writer wishes to thank the following members of the staff of the Food and Drug Administration for participating in the check grading of the sediment pads: V. W. Shover, W. J. Scanlon, J. A. Day, J. C. Akers,

* For report of Subcommittee C and action of The Association, see *This Journal*, 33, 50 (1950).

and F. J. Aull, Kansas City District; W. Vincent, L. Chernoff, F. Clark, R. Davidson, M. Gillham, R. Porter, and J. Roe, Denver District; R. C. Jordan, L. M. Levin, and L. J. Cramer, St. Louis District; E. C. Aderholdt, G. W. Sooy, and S. Weissenberg, of the Cincinnati District; F. J. Fiskett, J. E. Anderson, and C. R. Baeurlen, Minneapolis District; K. L. Harris, Division of Microbiology; J. O. Clarke, Division of Program Research; and the following for preparing standard sediment pads: H. W. Conroy, Kansas City District; G. E. Keppel, Minneapolis District; M. L. Porter, Denver District; F. H. Collins, Cincinnati District; H. D. Silverberg, St. Louis District; and A. H. Robertson, New York State Food Laboratory.

REFERENCES

(1) JOINER, C. R., *This Journal*, **32**, 324 (1949).
(2) "Changes in Methods of Analysis," *Ibid.*, **31**, 93 (1948).

REPORT ON EXTRANEOUS MATTER IN BEVERAGE MATERIALS

By F. A. HODGES (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

In line with the program advanced by the General Referee the following methods were subjected to collaborative study:

42.5—The method for mold count in canned citrus juice is considered satisfactory in the opinion of B. M. Moses, H. T. Hyde, J. T. Welch, A. H. Wells, and F. A. Hodges.

42.6—The method for the recovery of fly eggs and maggots in canned citrus juice is considered satisfactory in the opinion of B. M. Moses, A. H. Wells, R. E. O'Neill, and F. A. Hodges.

42.7—The method for the recovery of insect fragments and rodent contamination in canned citrus juice is considered satisfactory in the opinion of J. T. Welch, A. H. Wells, R. DeFrancesco, R. E. O'Neill, and F. A. Hodges.

42.8—The method for the extraction of rodent and insect excreta in tea, leafy crude drugs, and condiments should be deleted. It is replaced by the methods for marjoram, savory, and thyme.

42.9—The method for the recovery of insects, insect parts, and hairs in tea, leafy crude drugs, and condiments should be deleted. It is replaced by the method for marjoram, savory, and thyme.

42.10—The method for the recovery of filth in cocoa, imitation cocoa, cocoa substitutes, coffee and coffee substitutes is considered satisfactory in the opinion of J. T. Welch, A. H. Wells, R. E. O'Neill, and W. G. Helsel.

It is therefore recommended* that 4 of the above methods, viz., **42.5**,

* For report of Subcommittee C and action of The Association, see *This Journal*, 33, 50 (1950).

42.6, 42.7, and **42.10** be adopted as first action methods.

Thanks are due the following collaborators: B. M. Moses, Philadelphia District, U. S. Food and Drug Administration; J. T. Welch, Buffalo District; A. H. Wells, Los Angeles District; H. T. Hyde, San Francisco District; R. E. O'Neill, Atlanta District; R. DeFrancesco, Philadelphia District; K. L. Harris, W. G. Helsel, and F. A. Hodges, Washington, D. C.

REPORT ON EXTRANEOUS MATERIALS IN VEGETABLE PRODUCTS

By FRANK R. SMITH (Food and Drug Administration, Federal Security Agency, Washington 25, D.C.), *Associate Referee*

In accordance with the general plan for the re-evaluation of the methods in Chapter 42 collaborators were asked to express their opinions of the Method for Extraneous Materials in Vegetable Products. The collaborators were all analysts who had experience with the methods in question. The following two questions were asked each collaborator:

(1) Will the method clearly separate the filth elements from the food?
(2) Do you consider it a satisfactory method for the isolation of filth or detection of decay (mold)?

RECOMMENDATIONS*

Based on the replies of the collaborators (all of the Food and Drug Administration), the following recommendations are made:

That the following methods having been found satisfactory, be made first action:

42.60: Rot in Canned Tomatoes (deleting the phrase "or brush thru No. 30 sieve with stiff-bristle brush") has been found satisfactory by the following collaborators: Carl B. Stone and Wm. J. McCarthy, Cincinnati, Ohio; H. I. Macomber and Shirley M. Walden, Baltimore, Md.; Dorothy B. Scott, New York, N. Y.

42.61: Fly Eggs and Maggots in Tomatoes: V. W. Leland and Shirley M. Walden, Baltimore, Md.; Wm. W. Wallace, Seattle, Wash.; H. C. Van Dame and Frank H. Collins, Cincinnati, Ohio.

42.62: Insect Fragments in Tomato Products: Maryvee G. Yakowitz, Washington, D. C.; A. H. Wells, Los Angeles, Calif.; Robert T. Elliott, Seattle, Wash.; Juanita E. Breit, Cincinnati, Ohio.

42.64: Mold in Tomato Soup, Canned Spaghetti, Pork & Beans, etc.: Carl B. Stone and Wm. J. McCarthy, Cincinnati, Ohio; H. I. Macomber, Baltimore, Md.; Dorothy B. Scott, New York, N. Y.; Doris H. Tilden and Helen T. Hyde, San Francisco, Calif.

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* For report of Subcommittee C and action of The Association, see *This Journal*, **33**, 50 (1950).

42.66a: Rot in Pureed Infant Food: A. H. Wells, Los Angeles, Calif.; Kenton L. Harris, Washington, D. C.

42.67: Insects & Rodent Filth in Pureed Infant Food: A. H. Wells, Los Angeles, Calif.; Doris H. Tilden, San Francisco, Calif.; J. Frank Nicholson and Kenton L. Harris, Washington, D. C.

42.68: Fly Eggs & Maggots in Pureed Infant Food: Kenton L. Harris, Washington, D. C.

42.69: Weevils in Peas & Beans: Wm. C. Woodfin, Pittsburgh, Pa.; Kenton L. Harris, Washington, D. C.; J. Frank Nicholson, Washington, D. C.

Robert E. O'Neill and Phyllis B. Rokita, both of Atlanta, Ga. found the method less satisfactory than a revision which they suggested. This revision should be subjected to further study.

42.70, 42.71: Light and Heavy Filth in Canned Greens and Broccoli: One or both of these methods were found satisfactory by the following collaborators: C. R. Joiner, St. Louis, Mo.; Andrew G. Buell, San Francisco, Calif.; Kenton L. Harris and J. Frank Nicholson, Washington, D. C.

42.77: Sweet Corn Insect Filth: It is recommended that this method be deleted and the following inserted in its place:

Insect filth. Use a whole can as sample.

Microscopic Examination.—Place 200 g of well-mixed sample in 2-liter Wildman trap flask, with 20 ml of castor oil and mix well. Add sufficient hot tap H_2O (ca 50°) to fill flask. Let stand 30 min with occasional gentle stirring; then trap off into beaker the oil and water layer and any corn debris that may have risen into neck of trap flask. To dissolve the adhering oil, wash out neck of flask with hot alcohol. Add a little more (ca 10 ml) hot H_2O to flask, stir, let stand 10 min, and trap off again into same beaker. Heat trapped off portion to near boiling. Set a 3″ No. 8 or No. 10 sieve in 400 ml beaker and pour oil and water mixture onto sieve. Wash corn debris on sieve thoroly with hot alcohol. Filter material that passes thru sieve, washing beaker, sides of funnel, and paper thoroly with hot alcohol to dissolve the oil and speed filtration. Examine paper microscopically.

Macroscopic Examination.—Empty the residue of corn remaining in the bottom of the flask when trapping is completed onto a 5–8 inch No. 20 sieve. Place on sieve the remainder of corn from the can, portion-wise if necessary, and wash under the tap to remove starch and fine particles. Place residue on the sieve in a pan and examine under water for worm-eaten or rotten kernels and whole worms, heads, or large fragments.

This revised method was found satisfactory by the following collaborators: Kenton L. Harris, Washington, D. C.; Gloria Getchell and Sam H. Perlmutter, Minneapolis, Minn.; Shirley M. Walden, Baltimore, Md.; Carl B. Stone, Cincinnati, Ohio.

It is recommended that the following methods be included as screening or sorting procedures:

42.74, 42.75: Filth in Mushrooms, including the rapid test for maggots *This Journal*, **30**, 105 (1947).

It is recommended that the following methods be dropped, some of them being obsolete, and others needing further study.

42.59 Rot Fragments
42.63 Sand in Tomato Products
42.65 Dehydrated Tomato Products; Mold
42.66(b) Pureed Infant Food; Rot fragments
42.72 Insects in Asparagus
42.73 Mushrooms, decomposed material
42.76 Insects in Brussels Sprouts.

REPORT ON EXTRANEOUS MATERIALS IN DAIRY PRODUCTS AND EGGS

By KENTON L. HARRIS (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

Following the general plan for the re-evaluation of Chapter 42 methods, the methods for dairy products and eggs were subjected to collaborative study by asking each of several collaborators two questions: (1) Will the method clearly separate filth elements from the food? (2) Do you consider it a satisfactory method for the isolation of filth?

RECOMMENDATIONS*

42.11: Altho the method was considered reliable by R. E. Duggan, C. M. Joiner, and W. W. Wallace, these collaborators agreed that criteria other than staining have largely replaced the precise application of this technique. In addition S. H. Perlmutter, F. J. McNall, G. E. Keppel, and K. L. Harris find the staining criterion too ambiguous in view of the success they have found thru individual histological studies of authentic self-prepared material. It is recommended that this method be dropped.

42.12, 42.13: All of these procedures, as modified from time to time, have been applied successfully to various cheeses and cheese products. In the hands of a skilled analyst these methods can be combined into useful procedures. However, they are now undergoing extensive rearrangement and are, in their recent form, proposed for inclusion only as a set of suggested steps for cheese examination.

42.14: This method for mites has not been used for many years and is unnecessary under present commercial practices. It should be deleted.

42.15, 42.16, 42.17, 42.18, 42.19: These methods, as modified, warrant the same comments as given in **42.12, 42.13** above. They are recommended for adoption as the best available sorting tools.

42.21(a): This method is recommended by S. H. Perlmutter, W. W. Wallace, H. C. Van Dame, S. Fine, G. E. Keppel, and K. L. Harris for official action. The Associate Referee concurs.

42.21(b): Delete.

42.39, 42.40: These methods have shown considerable promise in the hands of the authors of the methods (respectively, K. L. Harris and R.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 50 (1950).

E. Duggan) but in the absence of any widespread use or collaborative data no recommendation can be made. It is suggested that they be included as useful tests and that a more definite recommendation be made when information becomes available.

The cooperation of the following collaborators greatly facilitated the preparation of this report: S. H. Perlmutter and G. E. Keppel, Minneapolis District; S. Fine, H. C. Van Dame, and F. J. McNall, Cincinnati District; R. E. Duggan, New Orleans District; C. M. Joiner, St. Louis District; W. W. Wallace, Seattle District; U. S. Food & Drug Administration.

REPORT ON EXTRANEOUS MATERIALS IN FRUIT PRODUCTS

By W. G. HELSEL (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

RECOMMENDATIONS*

It is recommended that sections **42.41** and **42.42** be deleted because they are essentially macroscopical procedures, and that the following be substituted as the method for frozen strawberries:

> Pulp the thawed berries thru cyclone with screen openings 0.027″ in diam. and mix thoroly (pour juice thru cyclone last). If necessary, remove air bubbles with suction or by mixing ca 100 g pulp with 3–5 drops of capryl alcohol. Mix thoroly and make mold count as in **42.57**.

Collaborators who have used this method and found it to be satisfactory are: W. V. Eisenberg, F. R. Smith, and M. G. Yakowitz of the Food and Drug Administration, Washington, D. C. The method is recommended for adoption as first action.

It is recommended that section **42.43** be deleted and the following substituted for it: "Make mold count as directed in **42.57**." Since section **42.57** is an official method no collaborative study should be necessary.

In section **42.44** the sentence beginning "Add 25–35 ml of castor oil" should be changed to read "Add 25–35 ml castor oil and mix thoroly. Add sufficient hot H_2O to bring oil layer into neck of flask." This method as changed was found to be satisfactory by F. A. Hodges, J. F. Nicholson, and M. G. Yakowitz, and it is recommended that it be made first action.

Sections **42.45** and **42.46** have been found to be satisfactory by F. A. Hodges, F. J. McNall, J. F. Nicholson, and F. R. Smith, of the Food and Drug Administration, and it is recommended that these two methods be made first action.

Section **42.47** is essentially a macroscopical procedure and should be deleted from the *Methods of Analysis*.

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* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 50 (1950).

Sections **42.48** and **42.49** should be included as recommended procedures.

Section **42.50** has been used extensively and has been found to be satisfactory by the following collaborators: F. J. McNall, Cincinnati, Ohio, J. Phyllis Skyrme, Boston, Mass., and J. F. Nicholson and M. G. Yakowitz of Washington, D. C. It is recommended that the method be made first action.

Section **42.52** should be made a procedure. This includes methods published *This Journal*, **30**, 97 (1947), for "Fruit Paste: Light Filth" and "Insect Heads in Fig Paste and Fig Slices."

Section **42.53** should be deleted and completely revised as soon as time permits.

The method for Insect and Rodent Filth in Jam and Jellies published in *This Journal*, **30**, 103 (1947), has been found to be satisfactory by collaborators F. A. Hodges and J. F. Nicholson. It is recommended that it be made first action.

The method for Mold in Cranberry Sauce, published *This Journal*, **32**, 116 (1949), has been found to be satisfactory by collaborators Catherine G. Cunningham, Rosa De Francesco, W. V. Eisenberg, F. R. Smith, and A. H. Tillson. It is recommended that it be adopted as first action.

It is recommended that the following methods published in *This Journal*, **30**, 103, 104, and 105 (1947), be deleted: Rot and Worm Fragments in Apple Pomace from the Manufacture of Apple Cider, Rot in Frozen and Canned Blueberries, Insect Infestation, Mold, and Sand or Soil in Raisins and Currants, Caterpillars in Lingon Berries, Insect and Rodent Contamination in Currant Pulp, Rot in Grape Pulp, and Filth in Dried and Canned Fruits (apples, peaches, pears, apricots, plums, and prunes).

The contributed paper entitled "The Relation Between Filth in Whole Figs and Filth Recovered from Fig Pastes," by J. T. Kelley and L. I. Pugsley, was published in *This Journal*, November 15, 1949.

The contributed paper entitled "A Method for the Improved Recovery of Insects and Insect Fragments from Fig Paste," by Doris Tilden, was published in a preceding number of *This Journal*, February, 1950.

REPORT ON FISH AND OTHER MARINE PRODUCTS

By ANDREW M. ALLISON (Food and Drug Administration, Federal Security Agency, Boston 10, Mass.), *Referee*

Chapter 24 "Fish and Other Marine Products" of the present 6th edition of *Methods of Analysis* has been subjected to study and there have

been additions of new material, deletions, corrections, and changes, for inclusion in Chapter 18 of the 7th edition of the new *Methods of Analysis*. The revisions follow closely the specific recommendations made by this Referee at the 1948 meeting of the A.O.A.C., *This Journal*, **32**, 331 (1949), and take into account amendments to the Constitution and By-laws, suggestions by the Secretary-Treasurer, and those of the Chairman of the Revision Committee. The several Referees whose subjects of study are a part of the chapter have been consulted. The recommended changes are as follows:

SUMMARY

Paragraphs **24.1**, Apparatus—Tentative, and **24.2**, Preliminary Treatment and Preparation of Sample—Tentative, from the present chapter have been rearranged and combined as one paragraph entitled "Procedure." Considerable analytical work on soft shell clams at the Boston District during the past year under the supervision of this Referee has demonstrated that apparatus, procedures, and methods now limited to oysters and scallops can be extended to include clams, so the name of this shellfish at appropriate places in the text has been added. In grinding and preparing homogeneous mixtures of fish and fish products, the use of the meat chopper, **24.1(d)**, has in the past been standard procedure. While no change is suggested in the use or description of the chopper from that in the present chapter, there has been added the sentence "As an alternate procedure, macerate and mix meat several min. in Waring Blendor."

In the 1948 Referee's report on the desirability of drained liquid studies was discussed and Subcommittee C, *This Journal*, **32**, 54 (1949), approved such work on shucked clams. The Boston District for the past year has been making a survey of the shucked soft shell clam industry. The studies are looking towards the establishment of definite proposals of food standards for this product. Drained liquid determinations and other methods of analyses needed for this work have been identical to those largely developed in the past in connection with the examination of shucked oysters. The oyster methods for the commonly used determinations have been found equally applicable to clams. Since the determination of drained liquid on the clams followed the essential details of that method as described for oysters in the Food Standards and was found to be satisfactory, it is the Referee's opinion that it should be accepted for clams without further study.

In connection with this same problem, State law enforcement authorities have recently called attention to the fact that their Courts criticized them for modifying the method in the Federal Raw Oyster Standards for the determination of drained liquid by not making the examination 15 minutes after packing, as stated in the Standards. It was further pointed out that a method without the time factor for the determination

of drained liquid as required in the Oyster Standards, if incorporated in the *Book of Methods*, would be most useful to State authorities and it would also be a method having the added prestige of official status.

In view of the above, the Referee recommends that "Drained Liquid—Official" in Shucked Oysters, Clams, and Scallops be included in the chapter on Fish and Other Marine Products of the forthcoming revised *Book of Methods*. The procedure will be essentially that described in S.R.A., F.D.C. 2. Revision of Definitions and Standards for Food, paragraph **36.10**(c) (2) (i) (ii), omitting, however, the requirement that the shucked shellfish be tested within 15 minutes after packing, and also omitting any reference to the limit of drained liquid.

Revisions include scallops along with oysters and clams, since there is no reason to believe that this shell fish would act differently upon application of this determination. It should also be mentioned that since the skimmer and method of obtaining drained liquid will be adequately covered in the new paragraphs, it will be to the interest of uniformity and the conserving of space in the revised paragraph to omit description of skimmer now in **24.1**(c) and to also to omit the technique of obtaining free liquid now given in **24.2**(f).

Paragraph **24.7** (Salt) II, With Calcium Acetate as Fixative—Tentative, appears to be an alternate method to paragraph **24.6** (Salt) I. Open Carius Method—Official. The first mentioned method has no reported advantages or special uses over the "Official" method. In line with our recommendation of last year, it is suggested that paragraph **24.7** be deleted.

At the 1948 A.O.A.C. meeting, Subcommittee C approved the method on Crude Fat—Acid Hydrolysis as official, first action, *This Journal*, **32**, 54 (1949). This method should be included as two paragraphs numbered and placed after the topic "Total Nitrogen—Official" in the 7th edition, and we concur with the Associate Referee's current recommendation that the method now be made official.

In connection with paragraphs **24.9, 24.10,** and **24.11,** Volatile Fatty Acids—Official, the Referee on Decomposition and Filth in Foods (Chemical Indices) reports that:

"Sections of the method covered by **24.9** and **24.10** will be changed to simplify the preparation of solution and standardization of distillation apparatus. Section **24.11** (b) under "Determination" will be changed to call for the computation of a volatile acid number. Reference to titration outfit will be retained. **24.11** (a) will be eliminnated. The next section of the method will be headed "Volatile Fatty Acids" and will include the determination of formic acid on a 2nd 200 ml portion of distillate and instructions for the chromatographic separation of the acids obtained in the first 200 ml of distillate (volatile acid number).

. . . We have sufficient collaborative data on chromatographic separation of volatile acids to recommend that the revised method be adopted—first action. . . . "

This Referee concurs in the above recommendations and suggests that the changes be made.

It is recommended that Indole in Shrimp, Oysters, and Crabmeat—Tentative, as approved by Subcommittee on Recommendations, *This Journal*, **31**, 51 (1948), be included as the last subject in Chapter 18 of the 7th edition revision of *Methods of Analysis*. The Referee on Decomposition and Filth in Foods and the Associate Referee on the specific subject now recommend that the tentative designation be changed to first action.

No work has been done on total solids in fish this year and it is recommended that the study be continued.

RECOMMENDATIONS*

It is recommended—

(1) That paragraphs **24.1** and **24.2**, *Methods of Analysis* 6th Ed., be combined under the title "Procedure."

(2) That minor changes be made in the revised section on "Procedure" to include clams where oysters and scallops are now mentioned, and to permit the use of the Waring Blendor as an alternate procedure.

(3) That new paragraphs be inserted in the chapter on Fish and Other Marine Products after the paragraph on Procedure, to be titled: "Drained Liquid—Official (Shucked Oysters, Clams and Scallops)."

(4) That paragraph **24.7** (Salt), be deleted.

(5) That Crude Fat—Acid Hydrolysis, first action, now be made official.

(6) That paragraphs **24.9, 24.10, 24.11,** Volatile Fatty Acids—Official, be changed in accordance with recommendations currently being made by the Referee on Decomposition and Filth in Foods (Chemical Indices).

(7) That Indole in Shrimp, Oysters, and Crabmeat—Tentative, under the same Referee on Decomposition and Filth in Foods, be changed to first action.

(8) That the study on Total Solids in Fish be continued.

REPORT ON TOTAL SOLIDS AND FREE LIQUID IN CLAMS

By MENNO D. VOTH (Food and Drug Administration, Federal Security Agency, Boston 10, Mass.) *Associate Referee*

It was the recommendation of Subcommittee C, *This Journal*, **32**, 54 (1949) that work be continued on methods for the determination of total solids in fish.

During the past year a considerable amount of work has been done on shucked soft-shell clams at the Boston District. In the course of this

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 52 (1950).

work it was possible to make some investigations on the determination of total solids. Duplicate determinations of total solids were made on 31 samples of shucked soft-shell clams using Method **24.3.** The average difference between duplicates was 0.14 per cent and the maximum difference was 0.49 per cent.

Comparable figures on oysters obtained in collaborative work in 1937 (*This Journal,* **20,** 412) show an average difference between duplicates of 0.09 per cent with a maximum of 0.39 per cent. These results confirm the obvious conclusion that the method described under **24.3** (6th edition, *Methods of Analysis*) is applicable to clams as well as oysters and there is no reason why clams should not be included under this paragraph.

No work on total solids in fish was done this year.

No work on ether extract in fish was done this year. No problems or questions have been received by the Associate Referee in regard to the method for ether extract in fish as reported in *This Journal,* **31,** 334 (1948). In view of the extensive work done on this method during the two previous years and the excellent collaborative results obtained, the Associate Referee recommends* that this method be adopted as official.

Subcommittee C also recommended that a study be made of methods for the determination of drained liquid in clams *This Journal,* **32,** 54 (1949).

In connection with other work at Boston District certain applicable data were obtained which show that a 2-minute draining period such as the present oyster standard prescribes for the determination of free liquid is also applicable to clams (Table 1).

TABLE 1.—*Per cent free liquid obtained after shucked soft-shell clams had been drained for various periods of time on factory skimmer*
(Figures obtained by draining gallons of clams 2 minutes on standard oyster skimmer as prescribed in Raw Oyster Standards)

DATE	DRAINING TIME ON FACTORY SKIMMER					
	1 MIN.	2 MIN.	3 MIN.	4 MIN.	5 MIN.	6 MIN.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
9- 9-48	6.68	5.69	5.39	5.76	4.88	
5- 3-49	11.20	6.38	5.68	5.40	4.82	
5- 5-49	6.10	5.80	5.80	5.00	4.80	
5-10-49	3.71	3.27	2.65	3.63	3.43	
5-12-49	2.99	2.79	2.96	1.59	1.82	1.67

The soft-shell clams used in this study were shucked by hand and washed in fresh water in various factories. After washing, the clams were placed on a factory skimmer which had perforations such as are prescribed in the Raw Oyster Standard. At the end of one minute a gallon of the clams was removed. At intervals of 2, 3, 4, 5, and 6 minutes, additional

* For report of Subcommittee C and action of the Association, see *This Journal,* 33 ,52 (1950).

gallons of the clams were removed from the skimmer. The free liquid of these various gallons of clams was determined within 15 to 30 minutes after removal from the factory skimmer by draining them two minutes on the standard oyster skimmer as prescribed in the Raw Oyster Standards. The results show that there is little change in the free liquid obtained after clams have been on the factory skimmer for 2 minutes.

All work on shucked soft-shell clams done at Boston District shows the close similarity between soft-shell clams and oysters. It would therefore follow that the skimmer prescribed in the oyster standards would work equally well with these clams.

REPORT ON SPICES AND CONDIMENTS

By SAMUEL ALFEND (Food and Drug Administration, Federal Security Agency, Kansas City, Mo.), *Referee*

No reports were submitted by the Associate Referees on Vinegar and Mustard. The studies recommended for this year should be continued. The subject of Vinegar should be re-assigned, since the Associate Referee will not be able to continue the work.

Arthur N. Prater was appointed very recently as Associate Referee on seeds and stems in chili pepper. He did not submit a report this year, but he has done some work on the subject. The studies should be continued.

MAYONNAISE AND SALAD DRESSINGS

Associate Referee Juanita E. Breit made a study of the method for fat, as applied to salad dressings low in oil. By modifying the manipulation slightly, she obtained excellent recoveries. She conducted a collaborative study which yielded results warranting her recommendation for adoption of the method as first action. The recommendation is approved.

VOLATILE OIL IN SPICES

The method was made official for sage and marjoram on the basis of good collaborative results, but was tentative for other spices because the results on some spices were erratic. This could be readily attributed in some instances to differences in the age of the portions analyzed by the several collaborators. There was a chance for loss of oil in some steps in the procedure.

Associate Referee N. Aubrey Carson has corrected these details, and has written more definite specifications for the method. His preliminary work showed that the method is not suitable for whole spices. The collaborative results on four different ground spices of widely varying oil content and physical characteristics demonstrated the reliability of the method. The values for specific gravity and Eugenol in the recovered oils were in satisfactory agreement. The Associate Referee's recommendation for adoption of these three methods as first action is endorsed.

The Referee has made suggestions to the Committee on Revision concerning editorial changes, and indicated that the following changes would be recommended formally:

The Preparation of Sample—Official, **33.1,** is really a procedure, and should be so designated.

With the adoption of the direct distillation method for Moisture, there is no longer any need for the inaccurate indirect method, **33.2,** and this method should be dropped.

The tentative method "Moisture—By Distillation with Toluene" should be made first action, on the basis of the collaborative work, *This Journal,* **24,** 83, 667 (1941), and subsequent general experience.

The tentative method for Cold-Water Extract in Ginger, **33.12,** appears to have no collaborative background, and the Referee is aware of no present use for it, so recommends that it be dropped.

The previous collaborative studies on the tentative methods for optical rotation, acid, and ester numbers and ketone and aldehyde in volatile oil (**33.19, 33.21, 33.24**) have not yielded concordant results, and their value does not appear to justify further work. They should be dropped.

Although not supported by collaborative work, Clevenger's work on "Volatile Oil and Resin in Ginger," **33.25,** *This Journal,* **19,** 98 (1936) and the usefulness of the method warrant adoption as first action. Reference to optical rotation, acid number, and ester number should be eliminated.

The paragraphs on Microscopic Examination should be designated as procedures (**33.29–33.33,** incl.).

Although the Referee can find no record of collaborative work on Ether Extract—Tentative in Prepared Mustard, **33.37,** the method is well-established and has been thoroughly tested, particularly by Hertwig and Palmore, *This Journal,* **7,** 170 (1923), and is sufficiently useful to warrant adoption as first action.

Since Chapter 21 has been recommended as first action, the method for Coloring Matter, **33.44,** should be made first action.

Preparation of sample under Mayonnaise and Salad Dressing, **33.46** should be a procedure.

The methods for reducing sugar and sucrose in mayonnaise and salad dressings, **33.48, 33.49,** and **33.50,** have been official, first action, for many years, pending further studies of the methods for preparation of sample and fat, *This Journal,* **24,** 66 (1942). Since this year's work on fat has been satisfactory, and there have been no adverse reports on the sugar methods since adoption, the methods for reducing sugars before and after inversion and for sucrose should be made official.

The methods for organoleptic examination and preparation of sample in vinegar, **33.58** and **33.59,** should be made procedures.

The work of Sanders and Henry, *This Journal,* **24,** 684 (1941), estab-

lished the limitations of the tentative methods for soluble and insoluble phosphoric acid in vinegar, **33.64** and **33.65.** In spite of the considerable variation in results caused by differences in temperature and time of ashing, the method is still one of the most valuable means for distinguishing between different types of vinegar. It is therefore recommended that the methods be adopted as first action.

The method for "Other Coloring Matters" in vinegar, **33.80,** should be made first action, since it refers to a chapter which has been made as first action.

The method for Polarization—Tentative, in vinegar should be made first action, on the basis of the collaborative work reported by Henry *This Journal,* **14,** 507 (1931).

Loughrey's work *This Journal,* **32,** 336 (1949) on **33.78** has confirmed the unreliability of the present official procedure, which should be dropped. Further work will undoubtedly lead to the adoption of either the bitartrate or the racemate method used for fruits.

H. J. Wichmann, the Referee on Metals in Foods, has suggested dropping **33.88,** Metals—Tentative, which is merely a reference to a whole chapter, and the Referee concurs.

The Committee on Revision proposes to label Dextrin (Qualitative) **33.89,** as a Procedure, and the Referee approves of this change.

The official method for Permanganate Oxidation Number, as published in *This Journal,* **32,** 102 (1949), did not include the directions in the second and third full paragraphs on p. 338, *Ibid.,* **32** (1949). These directions should be included in the final draft of the method, which is being recommended for adoption as official (final action).

RECOMMENDATIONS*

It is recommended—

(1) That "Preparation of Sample—Official," **33.1,** be made a procedure.

(2) That the method for "Moisture—By Drying with Heat—Tentative," **33.2,** be dropped.

(3) That the tentative method for "Moisture—By Distillation with Toluene," **33.3,** be made first action.

(4) That the tentative method for Cold-Water Extract (for ginger), **33.12,** be dropped.

(5) That the tentative method for volatile oil, **33.17,** with the slight modification described by the Associate Referee, be made first action, and no further work be done.

(6) That the tentative method for specific gravity of volatile oil, **33.18,** be made first action, and that no further work be done.

(7) That the tentative method for optical rotation of volatile oil, **33.19,** be dropped.

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* For report of Subcommittee C and action of the Association, see *This Journal,* **33,** 57 (1950).

(8) That the tentative method for acid number of volatile oil, **33.21,** be dropped.

(9) That the tentative method for ester number of volatile oil, **33.22,** be dropped.

(10) That the tentative method for Eugenol in volatile oil, **33.23,** be made first action, and that no further work be done.

(11) That the tentative method for ketone and aldehyde in volatile oil, **33.24,** be dropped.

(12) That the tentative method for volatile oil and resin in ginger, **33.25,** be made first action after deleting the references to optical rotation, and acid and ester numbers.

(13) That "Microscopic Examination—Tentative," **33.29–33.33,** incl. and "Prepared Mustard, Preparation of Sample," **33.34,** be made procedures.

(14) That the tentative method for ether extract in prepared mustard, **33.37,** be made first action, and the action be confirmed by collaborative studies.

(15) That in Total Nitrogen "Protein"—Official **33.38,** the phrase "Protein = N × 6.25" be dropped.

(16) That "Preparation of Sample—Tentative," **33.46,** be made a procedure.

(17) That the first action method "Reducing Sugars before Inversion" **33.48,** be made official.

(18) That the first action method "Reducing Sugars after Inversion" **33.49,** be made official.

(19) That the first action method for Sucrose, **33.50,** be made official

(20) That the tentative method for total fat in mayonnaise and salad dressing, **33.54,** with the slight modification described by the associate Referee, be made first action, and that no further work be done.

(21) That "Organoleptic Examination—Official," **33.58,** under Vinegars, be designated a procedure.

(22) That "Preparation of Sample—Official," **33.59,** under Vinegars, be designated a procedure.

(23) That the tentative method for soluble phosphoric acid in vinegar, **33.64,** be made first action, and that no further work be done.

(24) That the tentative method for insoluble phosphoric acid in vinegar, **33.65,** be made first action, and that no more work be done.

(25) That the tentative methods for polarization of vinegars, **33.81,** be made first action, and that no further work be done.

(26) That the official methods for determination of tartaric acid and tartrates in vinegar, **33.83** and **33.84,** be dropped.

(27) That the paragraph "Metals—Tentative," **33.88,** be deleted.

(28) That "Dextrin (Qualitative Test)—Tentative," **33.89,** be designated a procedure.

(29) That the first action method for permanganate oxidation number in vinegar, with the addition described in this report, be made official.

(30) That studies of methods for the detection of caramel in vinegar be continued.

(31) That studies of the determination of tartrates in vinegar be continued.

(32) That methods for the determination of free mineral acids in vinegar be further studied.

(33) That methods for determining sorbitol, and the usefulness of this method in detecting cider vinegar in wine vinegar, be studied.

(34) That the efficiency of the preliminary removal of fat in the official method for nitrogen in mayonnaise, **33.52,** be studied.

(35) That studies be initiated looking toward adoption of a method for the determination of ash in prepared mustard.

(36) That studies be continued on the determination of sugars in prepared mustard.

(37) That studies be continued of methods for the determination of volatile oils and other pungent principles in prepared mustard and mustard flour.

(38) That studies be made of methods for determining seed and stem content of chile peppers.

REPORT ON VOLATILE OIL IN SPICES

By N. AUBREY CARSON (U. S. Food and Drug Administration, Federal Security Agency, St. Louis, Mo.), *Associate Referee*

The accurate determination of volatile oil in spices has long been a problem of law enforcement chemists. In 1928 Clevenger (1) introduced a steam distillation apparatus with an oil trap by which a direct reading of the quantity of oil could be made. The oil could then be separated and examined for its characteristic physical and chemical properties. In 1932 Alfend and Mitchell (4) compared the then official ether extract method with Clevenger's method and with a modification of Cripp's and Brown's indirect method for volatile oil in cloves. The latter method determined volatile oil as the difference between the total volatile matter and the moisture. The ether extract method gave the lowest and least consistent results. The other two methods gave consistent results and checked each other fairly closely, but in Clevenger's method the oil was available for further examination. In 1932 Clevenger collected authentic data (2) on a variety of spices and in two successive years he submitted samples (3, 6) to collaborators. In 1934 Clevenger's method (5) was adopted as tentative by this Association. During subsequent years Clevenger collected authentic data (7–13,) on 20 different spices or related products.

He submitted 11 different spices to collaborators (14–21) with fairly good results. Clevenger concluded (21) that the variations in the results of his collaborators were due to two factors. One was the leakage of the volatile oil around the cold finger and the other was the lapse in time between analyses by different collaborators. He had previously shown (7, 10, 11, 22) that certain spices lost considerable volatile oil when held any length of time.

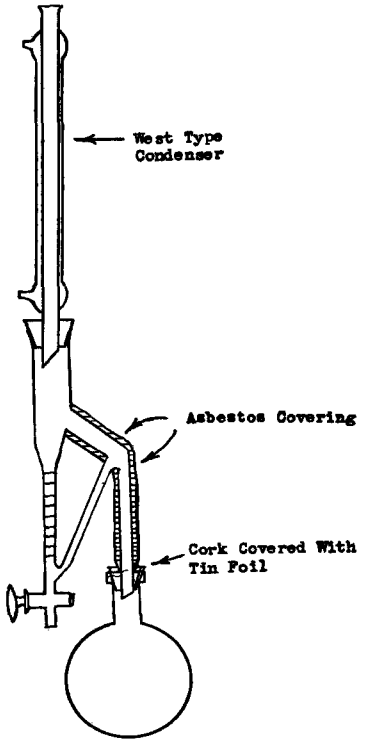

FIG. 1—Apparatus for the Determination of Volatile Oil.

EXPERIMENTAL

The purpose of this investigation was to determine whether or not with a few modifications the present tentative method for the determination of volatile oils in spices gave accurate and consistent results. Four spices (cloves, caraway, fennel, and coriander) were chosen as fairly representative of all the spices in volatile oil content. The samples were ground in an impact mill to pass a 20 mesh sieve and mixed thoroughly. Precautions were taken to prevent loss of volatile oil during grinding. The volatile oil content on these spices, both ground and whole, was deter-

mined. A West type condenser was used in place of the cold finger to prevent loss of oil around the condenser. Results which include refractive index and specific gravity are listed in Table 1.

TABLE 1.—*Experimental results on 4 representative spices*

SAMPLE	VOLATILE OIL % (v/w)	REFRACTIVE INDEX AT 20°C.	SPECIFIC GRAVITY AT 25/25°C.	EUGENOL % (v/v)
Whole Cloves	10.9, 14.8	1.5348, 1.5360	1.0590, 1.0635	84, 84
Ground Cloves	17.6, 17.4, 17.2	1.5320, 1.5318, 1.5343	1.0431, 1.0448, 1.0450	84, 84, 84
Whole Caraway	5.0, 5.4	1.4919	0.9269	
Ground Caraway	5.4, 5.4	1.4895, 1.4899	0.9117, 0.9122	
Whole Fennel	1.1, 1.1	1.5382, 1.5362	0.9660*	
Ground Fennel	1.6, 1.6	1.5370, 1.5372	0.9666*	
Whole Coriander	0.20, 0.30	1.4630, 1.4670	†	
Ground Coriander	0.28, 0.30	1.4642, 1.4640	†	

* Composite of two determinations.
† Insufficient amount of oil for analysis.

These results show that more oil was obtained from the ground cloves and fennel than from the corresponding whole spices. The results for volatile oil content in three of the four whole spices were inconsistent in comparison to those obtained from the ground spices. The lower yield of oil from some of the whole spices can be accounted for by the difficulty in completely removing the oil by this method. The inconsistencies in the results of the whole spices might be caused by the difficulty of obtaining a representative portion for analysis.

The method has been rewritten with as few changes as possible. A new illustration of apparatus is offered as a substitute for Figure 55 in the present tentative method (23). A West type condenser was substituted for the cold finger for reasons explained previously. The use of ether for washing the oil from the trap was omitted from the method because of the possible loss of a fraction of the oil (4) when the ether is evaporated. A preparation of sample was included in the method which requires that spices used be ground to pass a 20-mesh sieve.

Details of the method will appear in 7th Ed. *Methods of Analysis*. 1950.

COLLABORATIVE WORK

Portions of the well-mixed ground spices (clove, caraway, fennel, and coriander) were sent to collaborators. All collaborators were requested to keep samples refrigerated until used and to complete all analyses within three weeks. Specific instructions were given as to the amount of sample and the size of flask to use for each spice. The collaborators were required

TABLE 2.—*Collaborative results*

ANALYST	VOLATILE OIL % (v/w)	REFRACTIVE INDEX AT 20°C.	SPECIFIC GRAVITY AT 25/25°C.	EUGENOL % (v/v)
		Cloves		
A	17.6, 17.4, 17.2	1.5320, 1.5318, 1.5343	1.0431, 1.0448, 1.0450	84.0, 84.0, 84.0
B	16.0, 16.2	1.5322, 1.5322	1.0410, 1.0430	80.0, 80.0
C	17.6, 17.6, 17.6	1.5327, 1.5328, 1.5328	1.0452, 1.0454, 1.0452	84.0, 84.0, 84.0
D	17.3, 17.2	1.5320, 1.5322	1.0427, 1.0439	84.0, 83.5
E	17.2, 17.2	1.5315, 1.5315	1.0422, 1.0426	83.5, 83.5
Average	17.18	1.5323	1.0437	83.2
Std. Deviation	±0.51 or ±3.0%	±0.0007	±0.0011	±1.45
		Caraway		
A	5.4, 5.4	1.4895, 1.4899	0.9117, 0.9122	
B	5.5, 5.6	1.4890, 1.4890	0.9100, 0.9110	
C	4.8, 4.8	1.4871, 1.4867	0.9089, 0.9089	
D	5.4, 5.2	1.4880, 1.4884	0.9103, 0.9156	
E	5.5, 5.5	1.4868, 1.4868	0.9070, 0.9065	
Average	5.31	1.4881	0.9102	
Std. Dviation	±0.25 or ±4.70%	±0.0011	±0.0022	
		Fennel		
A	1.6, 1.6	1.5370, 1.5372	0.9666*	
B	1.6, 1.55	1.5385*	0.9690*	
C	1.3, 1.4	1.5341, 1.5351	0.9632, 0.9641	
D	1.5, 1.2, 1.4	1.5370, 1.5370	0.9673, 0.9669	
E	1.32, 1.35	1.5362, 1.5353	0.9613, 0.9584	
Average	1.44	1.5364	0.9646	
Std. Deviation	±0.13 or ±9.0%	±0.0013	±0.0033	
		Coriander		
A	0.28, 0.30	1.4642, 1.4640	†	
B	0.30, 0.33	1.4650*	0.862*	
C	0.30, 0.30	1.4631, 1.4629	†	
D	0.28, 0.28	1.4640, 1.4630	†	
E	0.33, 0.33	1.4636*	†	
Average	0.30	1.4637		
Std. Deviation	±0.020 or ±6.7%	±0.0007		

* Composite of two determinations.
† Insufficient amount of oil for analysis.

to determine specific gravity and refractive index on the volatile oil from each sample (24) and to determine Eugenol on clove oil (25). The collaborative results are listed in Table 2.

DISCUSSION OF RESULTS

Reasonable agreement on yield of volatile oil was obtained on the four spices examined. Two analysts with low yields of oil on fennel reported excessive foaming. A third analyst, C, used a smaller flask than specified on caraway, fennel, and coriander. This might be the reason for his low results on caraway and fennel. A wetting agent was added by Analyst C to coriander to facilitate distillation in a small flask. The Associate Referee used a wetting agent when distilling Fennel and coriander, which eliminated foaming and thus speeded up the distillation. The foaming and slowness of the distillation might be the cause for the few low yields. Reasonable agreement was obtained on the determination of specific gravity of the volatile oils examined. An insufficient amount of oil was obtained on the coriander to determine its specific gravity. Consistent results were obtained on the determination of Eugenol in clove oil between analysts.

ACKNOWLEDGMENT

The Associate Referee wishes to thank the following members of the United States Food and Drug Administration for their cooperation as collaborators: Adelyn D. Felmeister, New York District; Lloyd C. Mitchell, Minneapolis District; Hymen D. Silverberg, St. Louis District; and Floyd E. Yarnell, Kansas City District.

SUMMARY

A modification of the present tentative method for determination of volatile oil in spices was subjected to collaborative study and the results showed reasonable agreement between analysts. The physical tests, specific gravity and refractive index, which were determined on the volatile oils, showed good agreement between analysts. The determination of Eugenol in clove oil also showed good agreement between analysts.

RECOMMENDATIONS*

In view of the results obtained by this study and also by the previous work done by Clevenger, it is recommended—

(1) That the modification of the present tentative method for the determination of volatile oil in spices be adopted as first action.

(2) That the present tentative method for the determination of specific gravity in the oils of spices be adopted as first action.

(3) That the present tentative method for determination of Eugenol in the oils of spices be adopted as first action.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 57 (1950).

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(11) *Ibid*, **22**, 683 (1939).
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(14) *Ibid*, **18**, 417 (1935).
(15) *Ibid*, **19**, 411 (1936).
(16) *Ibid*, **20**, 410 (1937).
(17) *Ibid*, **20**, 602 (1937).
(18) *Ibid*, **21**. 435 (1938).
(19) *Ibid*, **21**, 566 (1938).
(20) *Ibid*, **22**, 598 (1939).
(21) *Ibid*, **25**, 700 (1942).
(22) *Ibid*, **17**, 285 (1934).
(23) *Methods of Analysis*, Ed. 6, **33.16**, p. 540 (1945).
(24) *Ibid*, **33.18** and **33.20**, p. 541.
(25) *Ibid*, **33.23**, p. 542.

REPORT ON FAT CONTENT OF LOW FAT SALAD DRESSINGS

By JUANITA E. BRIET (Food and Drug Administration, Federal Security Agency, Cincinnati, Ohio), *Associate Referee*

The present tentative method for the determination of fat in salad dressings, particularly those containing less than 50% fat, was subjected to further study in accordance with the recommendations of the Referee on spices and condiments.[1]

The Associate Referee ran a series of preliminary tests on low fat salad dressings using the present tentative method. Three extractions were made as specified in that method. A fourth extraction was then made, and on weighing it separately a significant amount of fat was recovered. This is shown by the following data:

Wt. of fat from 3 extractions (g)	0.3548	0.4982
Wt. of fat from fourth extraction (g)	0.0218	0.0297
% fat, 3 extractions	24.15	23.78
% fat, 4 extractions	25.64	25.20

[1] *This Journal*, **31**, 338 (1948).

Using the same sample the determinations were repeated with the following modification. After the addition of each ether, the Mojonnier tube was shaken violently for at least one minute. A fourth separate extraction was made as above, and the following data was obtained:

Wt. of fat from 3 extractions (g)	0.2780	0.3004
Wt. of fat from fourth extraction (g)	0.0005	0.0004
% fat, 3 extractions	28.90	28.88
% fat, 4 extractions	28.95	28.92

As a result of this work it was concluded that the principal difficulty with the method was the failure to shake vigorously enough to insure complete extraction of the fat, even when a fourth extraction was made. Emulsions formed by vigorous shaking were easily broken by centrifuging.

Because of the difficulty involved in preparing a stable salad dressing in the laboratory, the cooperation of two commercial firms was secured in obtaining samples for collaborative work. The Associate Referee was present while the products were being manufactured, and the samples used for collaborative study were taken from the production line.

Four jars of salad dressing were sent to collaborators with instructions. Results obtained by collaborators are reported in Tables 1 and 2. Table

TABLE 1.—*Fat in salad dressing No. 1*

ANALYST	SAMPLE NO.	FAT	DEVIATION
		per cent	*per cent*
1	A	27.35	+0.07
	A	27.29	+0.01
	D	27.22	−0.06
	D	27.32	+0.04
2	A	27.33	+0.05
	A	27.39	+0.11
	D	27.33	+0.05
	D	27.37	+0.09
3	A	27.33	+0.05
	A	27.25	−0.03
	D	27.30	+0.02
	D	27.19	−0.09
4	A	27.19	−0.09
	A	27.46	+0.18
	D	27.23	−0.05
	D	27.24	−0.04
5	A	27.08	−0.20
	A	27.17	−0.11
	D	27.25	−0.03
	D	27.21	−0.07
7	A	27.43	+0.15
	A	27.33	+0.05

TABLE 2.—*Fat in salad dressing No. 2*

ANALYST	SAMPLE NO.	FAT	DEVIATION
		per cent	*per cent*
1	B	20.11	−0.10
	B	20.11	−0.10
	C	19.90	−0.31
	C	20.03	−0.18
2	B	20.18	−0.03
	B	20.09	−0.12
	C	20.09	−0.12
	C	20.11	−0.10
3	B	20.26	+0.05
	B	20.22	+0.01
	C	20.23	+0.02
	C	20.16	−0.05
4	B	20.23	+0.02
	B	20.16	−0.05
	C	20.28	+0.07
	C	20.09	−0.12
5	B	20.35	+0.14
	B	20.22	+0.01
	C	20.42	+0.21
	C	20.29	+0.08
7	B	20.55	+0.34
	B	20.44	+0.23

3 compares the measures of dispersion and variation derived from the results shown in Tables 1 and 2. The theoretical fat content of duplicate samples A and D based on the formula used in making a 700 pound batch was 28.06%; for duplicate samples B and C, a 200 pound batch, 20.14%.

The results of Analyst 7 were obtained after samples A and B had separated on being refrigerated for four months.

TABLE 3.—*Comparison of results*

ITEM	PRODUCT 1	PRODUCT 2
	per cent	*per cent*
Arithmetic mean	27.28	20.21
Range, maximum	27.46	20.55
minimum	27.08	19.90
Standard deviation	0.09	0.15
Standard error	0.06	0.10
Probable error of the mean	0.01	0.02
Coefficient of variation	0.33	0.74
Coefficient of variance between Products 1 and 2	2.3	

DISCUSSION OF RESULTS

As samples A and D were alternately taken from a single mixed batch of salad dressing known as Product 1, and since Product 2 was sampled in the same way, to give samples B and C, all fat determinations for each product were treated together.

The data was compiled and analyzed to determine if any result had a significant deviation from the mean, and which consequently should be rejected according to the Pierce-Chauvenet criterion. No result was eliminated unless it were proven to be too far outside the permitted range as determined for the data as a whole.

Analyst No. 6 submitted the following determinations:

Product No.	*Sample*	*Fat (%)*
1	A	27.96
	A	27.73
	D	27.93
	D	28.43
2	B	21.46
	B	21.49
	C	20.88
	C	20.82

All of the above determinations had to be rejected as they were far too high by the Pierce-Chauvenet criterion. Analyst 7 had a range of 0.70% for Product 1 and 0.67% for Product 2, while the ranges for all the other results were 0.38% for Product 1 and 0.65% for Product 2. Errors were probably introduced through improper procedure, or some other trouble was encountered which should not be attributed to the method.

To compare the method as used on two different salad dressings, Table 3 was compiled to show the difference between the measures of dispersion and variation. The standard deviation for Product 1 was 0.09% as compared to 0.15% for Product 2. This increase in the deviation was due to the wider range and greater dispersion of results from the mean in the frequency distribution. The probable error of the mean for product 1 was, as a result, only one-half as much as that for Product 2; in other words, the accuracy of the mean is greater, which indicates the true mean has about a 50% chance of falling within the range $27.28 \pm 0.01\%$ for Product 1 as compared to a 50% chance for the true mean of Product 2 to be within the range $20.21 \pm 0.02\%$. The coefficient of variation for Product 2 was more than twice as large as that for Product 1. The smaller amount of fat in Product 2 enhances somewhat its coefficient of variation.

An analysis of the variation of the two distributions of data was made by finding the coefficient of variance, obtained by computing the difference between the natural logarithms (e) of the standard deviations. In perfectly fitting distributions, the coefficient of variation would be zero. To test the above coefficient (found to be +0.511), its standard error

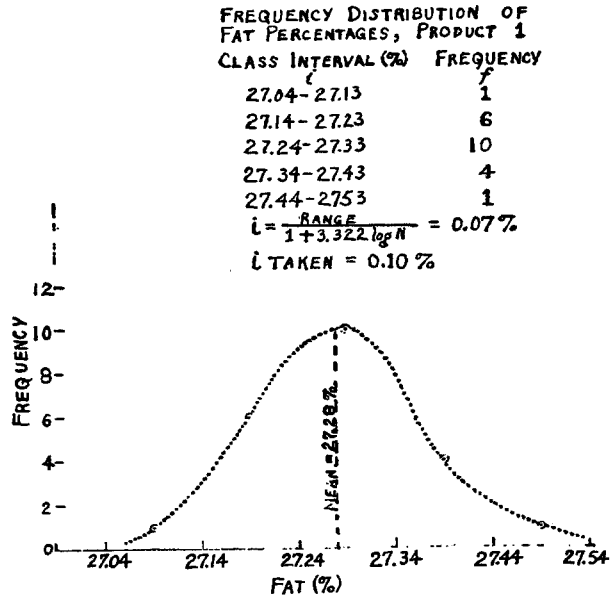

CHART 1.—Distribution of Percentages of Fat Found in Salad Dressing No. 1.

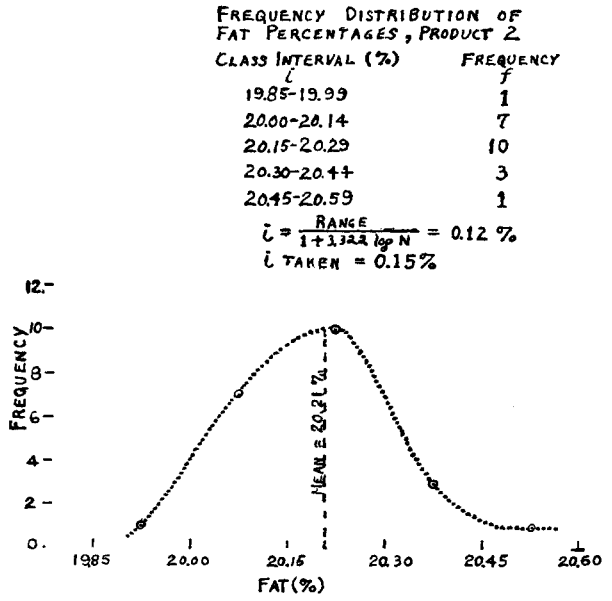

CHART 2.—Distribution of Percentages of Fat Found in Salad Dressing No. 2.

was computed and determined to be 0.218; hence, the coefficient deviates from zero by 2.3 times its standard error. Considering the number of results involved, this deviation is not to be considered too significant. The dispersion of fat values for Product 2, though greater, does not warrant considering the presence of sampling error or other important errors.

Charts 1 and 2 were constructed to show the resemblance between the two distributions. Though the class interval for Product 2 is $1\frac{1}{2}$ times larger than that for Product 1 (which was accounted for in the preceding discussion of the measures of dispersion and variation indicating a larger spread of value), the final curves closely resemble each other with respect to symmetry about the mean.

From the preceding discussion it can be concluded that the method used for extraction of fat from salad dressings with a low fat content is reliable. Details of the method are published in *Methods of Analysis*, 7th Ed. 1950.

ACKNOWLEDGMENT

The Associate Referee wishes to acknowledge the statistical study and interpretation of results made by Luther G. Ensminger of Cincinnati District. The cooperation of the following collaborators was appreciated: Frank H. Collins, Oro S. Keener, Mary McEniry, Aldrich F. Ratay, Jessie E. Roe, and J. Thomas Welch, all of the Food and Drug Administration.

RECOMMENDATION*

It is recommended that the method embodied in this report for the extraction of fat from mayonnaise and salad dressing be made first action.

REPORT ON METALS, OTHER ELEMENTS, AND RESIDUES IN FOODS

By H. J. WICHMANN (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Referee*

There has been a great change in the spraying of fruits and vegetables during the last five years. The change has been from inorganic to organic insecticides. Therefore, lead arsenate and the fluorine compounds are now no longer used or are used in greatly curtailed quantities. This poses the question what to do about the arsenic, lead, and fluorine sections of Chapter 29. This problem is particularly acute in those sections dealing with the rapid methods for apples and pears. The Referee has sounded available opinion about the feasibility of deleting these sections on the ground that the practical discontinuance of these toxicants renders the methods obsolete. The general reaction has been against deletion, because the future cannot be foretold with sufficient accuracy; any present un-

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 57 (1950).

foreseen difficulties with organic insecticides might bring the old favorites back into use. Certainly the general methods for arsenic, lead, and fluorine compounds should be retained in the Seventh edition, because they find usage in fields other than spray residues. In the light of all these considerations, the Referee considers it the better policy to retain these methods in the new edition.

Since the adoption of the lead and arsenic methods for the Sixth edition, certain streamlining changes have been proposed. They relate to changes in sample preparation for both arsenic and lead methods and to changes in *p*H in the lead determination. But the great change in the use of lead arsenate as a spray material resulted in a lack of interest and studies accordingly have not been continued. In the absence of collaboration on revised procedures changes cannot now be recommended. In fact it seems inadvisable to alter the arsenic and lead methods at this time in view of the general success with them as described in the Sixth edition. However, for the sake of these especially interested, the new ideas can be included as references in the manner already recognized in **29.3**. This appears to be the expeditious alternative to much collaborative work on details of such modifications at a time when they are of limited need.

ARSENIC

No changes are recommended other than making the bromate method, **29.6** to **29.10**, first action. There was sufficient collaboration on the bromate method when it was made tentative and nothing has appeared since to change the judgment of Referee and committee. Perhaps a reference to the Fahey, Cassil, Rusk method of sample preparation[1] which may be followed by a digestion of the "strip" solution, could be added in **29.8**. This plan is actually being followed with good results by one State chemist. No time or manpower is available for collaboration at this time. A reference to this plan will be sufficient for those who are interested.

CADMIUM

The collaborative results on cadmium in 1948 and 1949 are quite satisfactory even when comparatively large amounts of other metals, especially zinc, are present. The average error was kept below one microgram even when the interferences were in milligrams instead of micrograms. Such cases will not be encountered often in actual practice. The Referee recommends that the cadmium method be adopted, first action. Immediate continuation of collaborative work on cadmium is not recommended.

COPPER

There are two methods for copper in the sixth edition—one for a minimum of one milligram of copper and the colorimetric one for even smaller

[1] *This Journal*, **26**, 150 (1943).

amounts. In other words, we have one method for comparatively gross amounts and the other one for micro quantities. This is as it should be. The Referee wants to call attention to an error in the volumetric method that has been repeated in several revisions. The original collaborative work on the Low cuprous iodide-iodine copper method was done in 1931. The copper solution is made alkaline with ammonia and then acidified with acetic acid. The original directions called for acetic acid in *slight* excess. Too much acetic acid will bring about the liberation of iodine from potassium iodide. A slight excess was interpreted in the 1935, 1940, and 1945 editions to mean 1 ml. of 1+1 acetic acid. An occurrence in the Referree's laboratory this last year called his attention to the sluggishness of the reaction with 5 mg. of copper and 1 ml. of 1+1 acetic acid in a volume of 50 ml. It was found that 3 to 5 ml. of absolute acetic acid in a volume of 50 ml. was required for definite end points and quantitative results. Hence the Referee recommends that the 1 ml. of 1+1 acetic acid be changed to 10 ml.

DDT

The Associate Referee reported collaborative results in apple "strips" in 1948.[2] The results were fair but not outstanding. The Schechter-Haller colorimetric method and the one based on the determination of total organic cholrine seemed to have the edge on the others. Experience in the Referee's laboratory casts some doubt on the Stiff-Castello colorimetric method as being somewhat erratic and unreliable. This year attention was directed towards the determination of DDT in milk fat. Again, the results may be called fair but not highly accurate. Whether this is due to uneven distribution of the DDT in the fat, to difficulties of the methods themselves, or unfamiliarity of the analysts with the methods, it is difficult to say. The Referee recommends that both the colorimetric Schechter-Haller method for DDT and the total organic chlorine method for chlorinated hydrocarbons be adopted, first action.

FLUORINE

The general method for fluorine, **29: 22–28,** inclusive, has stood the test of time since it first was made tentative. The Referee therefore recommends that it be made official, and that the Rapid Method Restricted to Fluoride Residues on Apples and Pears be made first action. The fluoride determination other than in spray residues has become important in the last years because of the misuse of the rodenticide sodium-fluoro-acetate-1080.

LEAD

Because lead arsenate is rapidly becoming an obsolete insecticide no great interest has been manifested these last few years in improvements of the lead methods. Your Referee called attention last year to two sug-

[2] *This Journal*, **31**, 355 (1948).

gestions for streamlining the colorimetric lead method, but nothing has been done about it in the interim. The Referee believes that the general colorimetric method for lead should be made official even if nothing has been done about these newer ideas. Attention can be called to them by references for the benefit of those interested.

The churn method of stripping fruits and vegetables (Fahey, Cassil, and Rusk, *This Journal*, **26**, 150, (1943)) is similar in principle to the sample preparation of such products for DDT determinations. The only difference of consequence is in the nature of the reagents used. It is therefore in line with the trend of present day thinking on sample preparation. No time has been available to check collaboratively the conclusions of the original authors of the idea. However, the Referee has been assured by the Virginia State Chemist that at least 95 per cent of the lead and arsenic on even "difficult" apples is removed by the process. No information is available with respect to fluorine residues. The Referee believes himself justified in adding a reference to the Fahey, Cassil, and Rusk paper to sec. **29.49.**

MANGANESE

Manganese in Chapter 29 has long been handled by reference to other chapters. The Referee sees no reason for changing the practice. One of the methods to which reference is made is official in the Sixth Edition. It is recommended that the status of manganese in Chapter 29 be kept consistent with its status in the methods to which reference is made.

MERCURY

The Referee had hopes that this year's collaborative work on mercury would result in a basis for deletion of the present method and the adoption of a mercury method that could be applied to any food or biological material. At present it seems as if only the Associate Referee can get consistently successful results. More work on design of apparatus, selection of oxidizing reagent, and better description of method appears to be necessary. Further collaboration on mercury methods is therefore recommended.

The present method for mercury in the Sixth Edition is based on oxidation of organic matter by $KMNO_4$. This reagent is good for this job, but, unfortunately, it contains small and variable amounts of mercury, even in the better grades. No dependable method of mercury removal from $KMNO_4$ is known. It is therefore difficult to control blanks which becomes of extreme importance in the determination of very small quantities of the metal. Because of the difficulty with blanks and the old-fashioned method of mercury titration with dithizone solution, the Referee feels that the present method should be deleted, but unfortunately, he has nothing to put in its place. This is a very embarrasing but true statement of facts. Because of the competition for space in this Chapter, the Referee feels that it is better to have no mercury method at all in the next edition

than one in which the Association can have but little faith. Therefore, he recommends deletion. It is recommended that the mercury headline be retained with a list of references to the literature for the information of those interested.

SELENIUM

No changes are recommended in the section on Selenium.

TIN

The toxicity of tin is so low that a micro method for the element is hardly necessary in a chapter devoted to poisonous substances in or added to foods. The Referee understands that tin is of economic importance in the beverage industry. Therefore it is believed best that the determination of micro quantities of tin be confined to the Beverage chapter and that reference be made to it in Chapter 29, if and when a micro method is included in the Beverage chapter. In the meantime, it is recommended that the two methods in Chapter 29 be designated as first action.

ZINC

No report is being made on zinc determinations. That leaves the two methods, one for gross amounts and the other for micro quantities, in Chapter 29, as they are in the Sixth Edition. The only change recommended is the change from tentative to first action.

SODIUM-FLUORO-ACETATE—(1080)

During the last two years a number of cases of suspected poisoning of animals by sodium-fluoro-acetate, the war-inspired rodenticide 1080, have been encountered. There have also been numerous cases of very careless handling of 1080 in rat extermination campaigns wherein food supplies were in danger of contamination or were actually contaminated. Pharmacologists and toxicologists also were in need of a reliable method for the determination of this compound in biological material. All of this stimulated efforts to find a suitable method. It had been known for several years that inorganic fluorine could not be separated from organic fluorine compounds by ether extraction. This differentiates the fluoro-acetates from the homologous chloroacetates. Inorganic fluorine compounds are found in the animal body, particularly the bones. They are also found in foods, sometimes in minute amounts and sometimes in quite sizeable quantities, such as in marine products and tea. It was only when chromatographic technique was applied that progress became rapid. In fact, it is the keystone of the method. The collaborative results reported by this Association are probably the first ones ever made. While the recoveries are not 100 per cent, they are surprisingly good for a new method on a comparatively new product. They are certainly good enough to serve as a basis for the adoption of the method as first action, with the expectation that im-

provements will be made as time goes on. The Referee takes pleasure in recommending first action on this method for the determination of this dangerous residue when found in contaminated foods.

LOSS OF ORGANIC INSECTICIDES IN THE CANNING PROCESS

Two years ago Tressler[3] reported that DDT was lost in the canning process under some conditions and with certain foods. Britton and Fairing now report on canning losses suffered by methoxychlor, DDD, and parathion. In the case of methoxychlor, the losses are over 90 per cent and in the other two the losses taper down to about 50 per cent.

The data submitted clearly shows that some of these insecticides, or their breakdown products, have an influence on the flavor of the canned products. These results are important to both industry and regulatory officials. The next question concerns the nature of the breakdown products produced inside the can. One can hardly tell whether they are toxic until the structure is known. Their effect on methods of analysis is also speculative. The Referee thinks this line of research is not only extremely interesting but valuable as well and recommends it be continued.

ORGANIC INSECTICIDES OTHER THAN DDT

The Seventh Edition will contain a method for DDT. This is our initial venture into a new field of methodology. But there is a vast amount of work to be done on other insecticides already in use and others on the way. A method for BHC[4,5] in biological material will be published very soon. How much application it will have in spray residue work remains to be seen. Investigation on methods for the determination of chlordane and toxaphene are under way. The Referee had hopes for a report on the determination of parathion this year, but the campaign on DDT in milk interfered. The manufacturer of Aldrin (compound 118) offered a method for its determination at the San Francisco American Chemical Society meeting. Presumably it will be published soon.[6] A relative of parathion and a new "Bridge" compound, Dieldrin, are about ready for commercial production. It is expected that methoxychlor will have a much increased usage next year. Methods for its determination in spray residues and also in dairy products are expected to be published shortly. There is therefore no shortage in subjects for collaborative investigation. The Referee believes that Associate Referees should be named for the determination of parathion and possibly methoxychlor. These seem to be important insecticides and probably the ones which can be carried to the point of adoption before some of the others.

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[3] Tressler, C. J., Jr., *This Journal*, **30**, 140 (1947).
[4] *This Journal*, **32**, 751 (1949).
[5] *Ibid.*, **32**, 758 (1949).
[6] *Anal. Chem.*, **22**, 702 (1950).

RECOMMENDATIONS*

It is recommended—

(1) That the bromate method for arsenic be given first action status and that a reference to the Fahey, Cassil, Rusk method of sample preparation be added to **29.8.**

(2) That the method for cadmium reported by the Associate Referee be made first action and that further work on the element be discontinued to await call of the Referee.

(3) That the changes in the volumetric copper method discussed by the Referee in his report be made and that the two copper methods in the Sixth edition be designated as first action. Continuation of work on copper determination is recommended.

(4) That the colorimetric Schechter-Haller method for DDT in plant and animal products be adopted as first action, and that the total chlorine method for chlorinated hydrocarbons without specific designation be adopted as first action. Further work on these methods is also recommended.

(5) That the general method for fluorine be made official and the Rapid Method for Apples and Pears be made first action.

(6) That the general method for lead be made official and that it contain references to the stream-lining modifications suggested by Bambach and Buskey and by Synder.

(7) That the present mercury method be deleted and replaced by a list of references to mercury methods in the literature. It is also recommended that work on a mercury be continued.

(8) That the present tin methods be given first action status.

(9) That the two zinc methods be made first action methods.

(10) That the method for sodium-fluoro-acetate (1080) reported at the 1949 meeting, be adopted as first action.

(11) That the work on the determination of the amount and nature of losses of organic insecticides in the canning process be continued.

(12) That collaborative studies on the determination of parathion in foods be initiated.

(13) That studies on the determination of methoxychlor and especially its differentiation from DDT in foods particularly dairy products, be begun.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 54 (1950).

REPORT ON CADMIUM

By A. K. KLEIN (Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

As stated in the Associate Referee Report for last year, *This Journal* **32**, 349 (1949), the micro Cadmium method, as described in *This Journal*, **28**, 257 (1945), was submitted for collaborative study. Of five collaborators, one experienced difficulty in trial experiments and did not complete the work. The average value obtained by four collaborators on Sample I which contained 5.0 micrograms was 6.0; the average for Sample II which contained 20.0 was 18.5. The average deviation for Sample I was 1.0 microgram; for Sample II the deviation was 1.85 micrograms. Observations made by two collaborators regarding the extraction of cadmium by dithizone indicated the need for further study of the method.

The critical part of the method, one emphasized in both preceding reports is the purification of the carbon tetrachloride used in preparing the dithizone reagent. In order to depress the effect of zinc, which is usually present in foods and biological materials far in excess of cadmium, a dithizone extraction is finally made in the presence of 5% sodium hydroxide. Unless the carbon tetrachloride is free of certain impurities, probably sulfur compounds, cadmium is also repressed. In the 1948 study, collaborators were instructed to purify the carbon tetrachloride by refluxing with aqueous potassium hydroxide. The Associate Referee learned subsequently that this treatment does not always yield a sufficiently pure product. This fact may account for the irregularities observed by two of last year's collaborators. Carbon tetrachloride of sufficient purity may be obtained by refluxing the reagent with alcoholic potassium hydroxide. Except for this modification, the instructions submitted in the 1949 study were the same as those for 1948.

As in last year's study, two collaborative samples were submitted to the analysts (see Table 1). The samples consisted of 25 g. comminuted tomato, tested and found to be free of cadmium. Sample I contained 4.0 micrograms of cadmium along with 1700 micrograms of other added metals. Sample II contained 17.8 micrograms of cadmium along with 3400 micrograms of other metals. The interfering metals were the same as those described in last year's report. A practice sample of tomatoes containing no added Cd but all the other metals was also submitted.

Details of the method are published in the 7th Ed., *Methods of Analysis*, 1950.

Except for Collaborator A's analysis of Sample II, the results tend to be somewhat high. Apparently, the large amount of metal interferences, of which zinc comprises 60%, was not completely repressed. Obviously, the collaborative study constituted a rigorous test of the method. In the

determination of cadmium in samples containing the more usual amounts of other metals, better results may be expected.

The values reported on the practice sample indicate that when relatively large amounts of zinc are present, a small amount of this metal

TABLE 1.—*1949 Collaborative cadmium recovery*

ANALYST	MICROGRAMS CADMIUM				
	PRACTICE SAMPLE	SAMPLE I	DEV.	SAMPLE II	DEV.
A	Not reported	4.2, 4.4	0.2, 0.4	16.0, 16.7	1.8, 1.1
B	0	5.2	1.2	18.7	0.9
C	0.6, 0.8	4.8, 5.0	0.8, 1.0	17.8, 18.2	0, 0.4
D	0.7	4.5, 5.0	0.5, 1.0	18.3, 18.1	0.5, 0.3
Average		4.7	0.7	17.7	0.7

may be extracted and reported as cadmium. In such samples, oysters for instance, the final carbon tetrachloride extract, after the optical density is measured, should again be extracted with 5% sodium hydroxide. If the pink color persists the optical density is due to Cd. If the color is destroyed, the original optical density is due to zinc and may be ignored.

The determination of cadmium in the presence of zinc by extraction with dithizone from 5% sodium hydroxide as described in this procedure is not a new one. Fischer (*J. Angew. Chem.*, **50**, 919 (1937)) used the technique in determining cadmium in the presence of zinc in metals. More recently, Shirley *et al.* (*Anal. Ed.*, **21**, 300 (1949)) devised a similar method for the estimation of cadmium in biological material and foods.

It is recommended* that this method be given the status of first action.

The Associate Referee wishes to thank the following for their helpful collaborative assistance:

O. R. Alexander, American Can Co., Maywood, Ill.
P. Somerville, J. H. Heinz Co., Pittsburgh, Pa.
H. O. Fallscheer, H. M. Bollinger, A. Kleinman, L. M. Ferris, and T. J. Klayder of the Food and Drug Administration.

REFERENCE

This Journal, **28**, 257 (1945); **32**, 349 (1949) and final report. *Anal. Chem.*, **21**, 3C0 (1949).

* For report of Subcommittee Cancel action of the Association, see *This Journal*, **33**, 54 (1950).

REPORT ON MERCURY

By A. K. KLEIN (Food & Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

In last year's report, *This Journal*, 32, 351 (1949), the Associate Referee stressed the need for an almost complete destruction of organic matter if the entire sample digest is used for the extraction of mercury, a necessity when only a very small amount of mercury is present. A special apparatus consisting of both a modified Soxhlet unit for trapping water and volatile acids and the usual reflux condenser, was described as meeting this requirement. As more nitric acid was needed in this method of sample preparation than is used by other workers, who do not effect a complete digestion with acids, the final digest was reduced to an acidity of approximately 1 *N* by adding 50% sodium hydroxide. The solution was then refluxed with hydroxylamine hydrochloride to destroy oxidizing substance which if present would decompose the dithizone reagent used in the subsequent extraction of mercury.

During the current year, this method of sample preparation was successfully used by the Associate Referee on a variety of food products. Mercury was isolated from interfering metals and evaluated by either of two procedures: The one devised by Laug,[1] or a modification of the one described by Cholak and Hubbard.[2] Laug separates mercury from possible traces of copper by treating the mixed dithizonates with a hydrochloric acid solution of potassium bromide. Copper remains as the dithizonate, dissolved in chloroform, and is discarded. Mercury is retained as the potassium bromide complex, which is then converted to mercuric bromide by adding concentrated disodium phosphate buffer so that a *p*H of about 6 is obtained. It is then evaluated as the dithizonate. The Associate Referee was unable to remove completely all metals which react with dithizone from either the potassium bromide or phosphate reagents. Although the effect of the residual metals was very small and, moreover, was incorporated in the standard reference curve, the preparation of the two reagents was somewhat troublesome. Winkler,[3] and Cholak and Hubbard,[2] separate mercury from copper by treating the mixed dithizonates with an acid solution of sodium thiosulfate. Again, copper remains unchanged as the dithizonate, dissolved in chloroform, and is discarded. The aqueous solution containing mercury as the thiosulfate complex, is then heated with potassium permanganate to destroy the cŏmplex. Mercury is then evaluated as the dithizonate or as the beta-naphthyl analogue. However, as reported by Hubbard,[4] potassium permanganate contains small but measurable amounts of mercury. The As-

[1] *This Journal*, 25, 399 (1942).
[2] *Anal. Chem.*, 18, 149 (1946).
[3] *Methods of Analysis, A.O.A.C.*, 6th ed., 29.54 (1945).
[4] *Anal. Chem.*, 12, 768 (1940).

sociate Referee overcame the need for this reagent by destroying the thiosulfate complex, with sodium hypochlorite. The excess of this reagent was then decomposed with hydroxylamine hydrochloride. No heating was needed to effect either of the two last reactions.

As a test of the procedure during the past year collaborators were supplied with samples of comminuted tomato to which was added a known amount of mercury as the chloride along with much greater amounts of other metals. The submitted results were on the whole unsatisfactory. Except for the results of one collaborator, they tended to be low. The fault obviously lay in the sample preparation as the mercury values determined by the two methods of isolation and estimation agreed quite well. Several collaborators suspected that mercury was lost by volatilization during the final stage of digestion.

After receiving the various reports, the Associate Referee conducted experiments to determine at which step of the method mercury may be lost. A solution of 125 mmg. of mercury as the chloride in 125 ml. of water and 20 ml. each of sulfuric and nitric acids was distilled and collected in 25 ml. fractions. The distillation assembly was of standard taper Pyrex glass with thermometer extending into the solution. The following table of results indicates the amounts of mercury found in the various distillates.

FRACTION NUMBER	BOILING RANGE °C.	MERCURY FOUND
		mmg.
1	108–111	None
2	111–114	None
3	114–120	None
4	120–130	0.9
5	130–166	3.0

These data indicate that no mercury will be lost during the preliminary digestion of a sample in an apparatus equipped with an efficient reflux condenser, a conclusion proved by the following experiment. A 75 g. portion of apricot "mush" (1 part dried fruit comminuted with 3 parts by weight of water) containing 125 mmg of added mercury as the chloride was heated under reflux with 40 ml of mitric and sulfuric acids (1+1). The evolved nitrogen dioxide fumes were collected in water kept alkaline by the addition of 50% sodium hydroxide. After cessation of nitrogen dioxide, the alkaline trap solution was acidified, boiled under reflux, and tested for mercury. No mercury was found. This experiment was repeated with 50 g. of apples as base. Again no mercury was found in the evolved nitrogen dioxide. After the copious formation of NO_2 had lessened, the digestion was completed, and the digest then heated to fuming for 15 minutes. During this last treatment of the digest, the vapors from the

condenser were trapped in a dilute solution of sodium hydroxide. No mercury was found in this solution. This experiment was repeated with a solution of 125 mmg. of mercury as the chloride dissolved in 40 ml. of nitric and sulfuric acids (1+1). Again no mercury was found in the vapors leading from the condenser. Yet, when a solution of 10 mmg. of mercury in 15 ml. of sulfuric acid was fumed for 0.5 hr., diluted with water, and then adjusted to a normality of 1 with 50% sodium hydroxide, a recovery of only 80% was obtained. This experiment was repeated and the same low recovery resulted. But if instead of neutralizing in part with sodium hydroxide, the solution was diluted to 400 ml. to effect an acidity of 1 *N*, the recovery was quantitative. Obviously then, the low recovery obtained when the acid digested is adjusted with alkali is due to repression of the dithizone extraction by the relatively high concentration of sodium sulfate. As proof of this reasoning, it was observed that a recovery of only 73% could be obtained from 10 mmg. of mercury dissolved in a 1*N* sulfuric acid solution of 10% sodium sulfate, even though 3–10 ml. extractions of 5.5 mg./L dithizone were employed. A recovery of only 75% was also obtained from this concentration of sodium sulfate even when the acidity was lowered to 0.5 *N*. The effect of potassium sulfate was even more pronounced. In two tests with three 10 ml. dithizone extractions (5.5 mg./L) of 10 mmg. of mercury, dissolved in 300 ml. of 10% potassium sulfate at 1 *N* acidity, recoveries of only 20 and 30% were obtained.

Quite likely, therefore, the low values reported by the collaborators were due to the repressive effect of sodium sulfate rather than to loss by volatilization. The Associate Referee observed losses of mercury by volatilization only when dry cereal was heated with a mixture of concentrated nitric and sulfuric acids. In these instances, the initial reaction was so violent that the evolved nitrogen dioxide fumes carried liquid vapors of the digest uncondensed through the entire reflux assembly. When the cereal was diluted with water prior to heating with the mixed acids, the reaction rate was diminished and no loss of mercury was observed.

In next year's collaborative mercury study, the analysts will be instructed to effect a careful though complete acid digestion of the organic material, destroy residual oxides with urea, dilute the digest to 400 ml., with water, and then carry through the extraction procedure. At this dilution, the resulting acidity is approximately 1*N*. However, even at an acidity corresponding to 2.0 *N*, mercury may be quantitatively extracted with dithizone if the number of extractions is increased.

The instability of mercuric dithizonate to strong light has been noted by many workers. Somewhat recently, Reith and Gerritsma,[5] observed that when the final extraction of mercury by the dithizone reagent is made from an aqueous solution containing 1 ml. of 30% acetic acid per 10 ml. aqueous volume, enough of the acid is extracted by the chloroform

[5] *Rec. trav. chim.*, 64, 41 (1945).

solution of dithizone to stabilize the mercury complex. The Associate Referee found this to be true not only of the dithizonate but of the mercury beta-naphthyl analogue as well. In the presence of acetic acid, the optical densities of both mercury complexes remained constant even when exposed for 5 minutes to the comparatively strong light source of a neutral wedge photometer. The addition of acetic acid to the final aqueous solution to be evaluated for mercury by the dithizone procedure is therefore recommended.

Cholak and Hubbard[2] have demonstrated the superiority of di-beta-naphthylthiocarbazone (DN) to dithizone in the colorimetric determination of mercury. The color of the mercury complex of DN in chloroform is bright red, whereas the color of mercuric dithizonate is salmon pink. It is therefore much easier to approximate visually the amount of mercury in the initial extraction of the sample solution when DN reagent is used. Unfortunately, however, this reagent is not yet commercially available in sufficient purity. A method of preparing and purifying the compound is described by Hubbard and Scott.[6] A discussion of the purification of the reagent is also given by Cooper and Kofron.[7]

It is recommended*—

That the micro mercury method as applied to the analysis of foods and biological products be further studied.

REPORT ON DETERMINATION OF DDT IN PLANT AND ANIMAL MATERIALS

By R. H. CARTER (Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, Beltsville, Md.), *Associate Referee*

In 1948 the Associate Referee (1) recommended two methods for the determination of DDT residues in plant and animal material—Umhoefer's (2) procedure for determining the total organic-chlorine content, and the colorimetric method of Schechter *et al.* (3) based on the nitration of the compound and the development of a blue color by the addition of sodium methylate. Details of sample preparation and some modifications (4, 5, 6) of the methods to permit more accurate determinations were given.

In order to check the methods, in 1949 samples containing known amounts of DDT were prepared and submitted to a number of collaborators for analysis. The samples were taken from stock solution prepared by adding 40 mg. of DDT containing the *p,p'* and *o,p'* isomers (3:1) to 1 kg. of pure butter oil. Results of analyses of this material are reported in Table 1.

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[6] *J. Am. Chem. Soc.*, 65, 2390 (1943).
[7] *Anal. Chem.*, 21(9), 1135 (1949).
* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 54 (1950).

TABLE 1.—*Analyses of 1949. Collaborative Sample No. 2, DDT in butter oil*

COLLABORATOR	DDT (P.P.M.) CALCULATED FROM—	
	ORGANIC-CHLORINE DETERMINATIONS	COLORIMETRIC DETERMINATIONS
1	37.3, 36.0	—
2	37.0	46.0
3	37.6	—
4	—	39.0
5	—	33.9
6	45.0	—
7	—	47.5
8	—	36.0
9	—	45.1
10	33.6	—
11	—	43.8
12	—	44.3
13	—	41.9
14	—	44.3
15	—	41.2
16	—	37.5

All analyses were obtained by the methods outlined by Carter (1), except the second figure for Collaborator 1, which was obtained by the method of Wichmann, *et al.* (6), and the figure for Collaborator 16, which was obtained by the method of Clifford (7). If more than one determination was reported, the average is recorded in the table. Colorimetric determinations were reported as being made by spectrophotometer, neutral wedge photometer, and photoelectric photometer. Chlorine determinations were reported as being made by the Volhard titration method and by electrometric titration.

The results reported are only those which came within 20 per cent of the added amount. Four chlorine determinations and three colorimetric determinations were very much outside this range.

The results did not indicate a marked superiority in accuracy of one procedure over another. The variations can probably be attributed to lack of experience and unfamiliarity with the techniques by some of the collaborators.

The following collaborators assisted in this work:

Food and Drug Administration, Federal Security Agency—
Paul Clifford and A. K. Klein, Washington, D. C.
Anthony V. Daly and Paul A. Mills, San Francisco, Calif.
Sam Perlmutter, Minneapolis, Minn.
Shirley M. Walden, Baltimore, Md.
T. J. Klayder, H. P. Eiduson, and L. W. Ferris, Buffalo, N. Y.
Bureau of Entomology and Plant Quarantine, U. S. Department Agriculture—
H. D. Mann, P. E. Hubanks and Paul Giang, Beltsville, Md.
Jack E. Fahey, Vincennes, Ind.

W. E. Westlake, Yakima, Wash.
H. V. Claborn, Kerrville, Tex.
American Can Company: O. R. Alexander, Maywood, Ill.
Maine Agricultural Experiment Station: Stanley Getchell, Orono, Maine
H. J. Heinz Company: L. W. Mayer, Pittsburgh, Pa.
Canada Department of National Health and Welfare, Food and Drug Division: L. I. Pugsley, Ottawa, Ontario.

CONCLUSIONS AND RECOMMENDATIONS

Since the colorimetric procedure based on the reaction between sodium methylate and nitrated DDT is not subjected to interference from other insecticides except TDE, so far as known, it is recommended that this method as described in references (1), (3), (5), and (6) be adopted as official first action for the determination of DDT in animal and vegetable products. The colorimetric procedure as described by Clifford (7), using lipase hydrolysis of fats, although of limited application, also appears to be satisfactory.

The determination of organic chlorine as described in references (1, 2, and 4) is not specific for DDT, but appears to be reliable. It is recommended* that this procedure be adopted as official first action for the determination of the organic-chlorine content of animal and vegetable products where specific and confirmatory methods are not available.

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(5) SCHECHTER, M. S., POGORELSKIN, M. A., and HALLER, H. L., *Ibid.*, **19**, 51 (1947).
(6) WICHMANN, H. J., PATTERSON, W. I., CLIFFORD, P. A., KLEIN, A. K., and CLABORN, H. B., *This Journal*, **29**, 188 (1946).
(7) CLIFFORD, P. A., *Ibid.*, **30**, 337 (1947).

REPORT ON INSECTICIDES IN CANNED FOODS

A STUDY OF THE EFFECTS OF PROCESSING ON SPRAY RESIDUE

By W. A. BRITTIN (*Associate Referee*) and J. D. FAIRING (Contribution from the Food Laboratory of the Beech-Nut Packing Co., Canajorie, N. Y.)

An investigation was undertaken at the invitation of the A.O.A.C. to determine (1) whether an insecticide is decomposed upon processing and storage and (2) the nature of the decomposition products and their effects on the analytical determinations of the insecticide residue. At the

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 54 (1950).

present time it has been possible to get definite information on only the first part of this study and the second part is still under investigation.

In order to obtain food products containing known amounts of insecticides for this work, 100 lb. batches of the product under consideration were prepared in the normal manner. At the beginning of the cooking operation, the proper amount of insecticide, dissolved in 100 milliliters of 95% ethyl alcohol, was added. The product was puréed to insure uniform

TABLE 1.—*Products studied, and concentration in p.p.m. of insecticide added*

PRODUCT	PARATHION	METHOXYCHLOR	DDD	BHC*	CLORDANE*
Applesauce	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1				0.01, 0.1, 1
Apricots	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1				
Green Beans			0.1, 0.5, 1, 2, 5		
Peaches	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10	2, 5, 10		2, 5, 10	0.01, 0.1, 1
Pears	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1				0.01, 0.1, 1
Peas	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, 10	2, 5, 10		2, 5, 10	
Spinach	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1				
Veg. & Beef Soup	0.01, 0.02, 0.05, 0.1, 0.2, 0.5, 1				

* Flavor tests only.

dispersement of the insecticide in the sample and was then packed under vacuum in glass containers.

Table 1 lists the products studied and the concentrations of the insecticides used. Within a period of three weeks after processing, the products were analyzed by the appropriate method for the added insecticides. The results of these analyses are shown in Table 2. The methoxychlor analyses shown in the table were made by the method of Fairing and Warrington (1) and the results were checked, with excellent agreement, by the Schechter-Haller (2) procedure for DDT. The DDD analyses were made by the Schechter-Haller method and the parathion determinations by the method of Averell and Norris (3). All analyses were run in

TABLE 2.—*Loss of added insecticide following processing*

INSECTICIDE	PRODUCT	CONCENTRATION ADDED	CONCENTRATION FOUND AFTER PROCESSING	RETENTION	LOSS
		p.p.m.	*p.p.m.*	*per cent*	*per cent*
Methoxy-clor	Peaches	2.0	0.165	8.25	91.75
		5.0	0.231	4.62	95.38
		10.0	0.440	4.40	95.60
	Peas	2.0	0.099	4.95	95.05
		5.0	0.131	2.62	97.38
		10.0	0.436	4.36	95.64
DDD	Green Beans	0.1	0.05	50	50
		0.5	0.21	42	58
		1.0	0.56	56	44
		2.0	0.85	43	57
		5.0	2.63	53	47
Parathion	Applesauce	0.01	0.000	0	100
		0.02	0.002	10	90
		0.05	0.023	46	54
		0.1	0.044	44	56
		0.2	0.046	23	77
		0.5	0.165	33	67
		1.0	0.278	27.8	72.2
	Apricots	0.01	0.014	140	0
		0.02	0.018	90	10
		0.05	0.030	60	40
		0.1	0.043	43	57
		0.2	0.083	41.5	58.5
		0.5	0.200	40	60
		1.0	0.459	45.9	54.1
	Peaches	0.01	0.006	60	40
		0.02	0.018	90	10
		0.05	0.040	80	20
		0.1	0.082	82	18
		0.2	0.147	73.5	26.5
		0.5	0.361	72.2	27.8
		1.0	0.745	74.5	25.5
		2.0	0.950	47.5	52.5
		5.0	2.52	50.4	49.6
		10.0	4.04	40.4	59.6
	Pears	0.01	0.007	70	30
		0.02	0.006	30	70
		0.05	0.006	12	88
		0.1	0.030	30	70
		0.2	0.058	29	71
		0.5	0.156	31.2	68.8
		1.0	0.298	29.8	70.2

TABLE 2.—*Continued*

INSECTICIDE	PRODUCT	CONCENTRATION ADDED	CONCENTRATION FOUND AFTER PROCESSING	RETENTION	LOSS
		p.p.m.	*p.p.m.*	*per cent*	*per cent*
	Peas, not retorted	0.01	0.000	0	100
		0.02	0.008	40	60
		0.05	0.018	36	64
		0.1	0.042	42	58
		0.2	0.036	18	82
		0.5	0.229	45.8	54.2
		1.0	0.352	35.2	64.8
	Peas, retorted	0.01	0.003	30	70
		0.02	0.004	20	80
		0.05	0.004	8	92
		0.1	0.032	32	68
		0.2	0.071	35.5	64.5
		0.5	0.159	31.8	68.2
		1.0	0.347	34.7	65.3
		2.0	0.568	28.4	71.6
		5.0	1.10	22	78
		10.0	2.64	26.4	73.6
	Spinach	0.01	0.005	50	50
		0.02	0.000	0	100
		0.05	0.023	46	54
		0.1	0.030	30	70
		0.2	0.037	18.5	81.5
		0.5	0.097	19.4	80.6
		1.0	0.173	17.3	82.7
	Vegetable and Beef Soup	0.01	0.004	40	60
		0.02	0.013	65	35
		0.05	0.007	14	86
		0.1	0.011	11	89
		0.2	0.091	45.5	54.5
		0.5	0.114	22.8	77.2
		1.0	0.338	33.8	66.2

duplicate, and when disagreement was found additional determinations were made. In instances where further analyses were run on products containing parathion and methoxychlor six months and a year after processing there was found to be no change in the residue content within the limits of experimental error.

In addition to these chemical analyses, organoleptic tests were made by a panel of testers. In the case of benzene hexachloride and methoxychlor these taste tests were made at various periods after processing, as shown in Table 3. DDD and chlordane were run only once after processing,

TABLE 3.—*Fraction of voters detecting off-flavor at various periods after processing*

INSECTICIDE	PRODUCT	CONCENTRATION ADDED	OFF-FLAVOR 4 WEEKS	OFF-FLAVOR 6 WEEKS	OFF-FLAVOR 8 WEEKS	OFF-FLAVOR 10 WEEKS	OFF-FLAVOR 52 WEEKS
		p.p.m.					
B. H. C. 98% gamma	Peaches	0	0.1	0.6	0.1	0.0	0.3
		2	0.9	1.0	0.8	0.9	0.1
		5	0.7	—	—	—	0.4
		10	1.0	—	—	—	0.7
B. H. C. 98% gamma	Peas	0	—	—	0.1	0.3	0.3
		2	0.4	0.8	1.0	0.9	0.5
		5	0.8	0.9	1.0	—	0.5
		10	1.0	1.0	1.0	—	0.6
Methoxychlor	Peaches	0	0.1	0.6	0.1	0.0	—
		2	0.4	0.3	0.4	0.4	
		5	0.6	0.5	0.5	0.4	—
		10	1.0	0.7	0.8	0.7	—
Methoxychlor	Peas	0	—	—	0.1	0.3	—
		2	0.3	0.1	0.0	1.0	
		5	0.1	0.3	0.1	0.9	—
		10	0.8	0.9	0.8	0.9	—

TABLE 4.—*Fraction of voters detecting off-flavor after processing*

INSECTICIDE	PRODUCT	CONCENTRATION ADDED	OFF-FLAVOR
		p.p.m.	
DDD	Green Beans	0	0.0
		0.1	0.0
		0.5	0.3
		1.0	0.7
		2.0	0.4
		5.0	0.9
Chlordane	Applesauce	0	0.8
		0.01	0.0
		0.1	0.3
		1.0	0.5
Chlordane	Peaches	0	0.0
		0.01	0.2
		0.1	0.2
		1.0	0.4
Chlordane	Pears	0	0.3
		0.01	0.6
		0.1	0.7
		1.0	0.5

as shown in Table 4. Taste tests were also run on the products containing parathion; however, only a small panel was used and the results showed no significant variation between the control and initial concentrations up to one part per million which was the highest concentration presented to the panel.

Table 2 also shows the per cent of the insecticide lost or broken down after processing as indicated by the analytical methods employed. Since the greatest single source of error in this work is the chance of in-

TABLE 5.—*Average loss, for all products, of added insecticide on processing*

INSECTICIDE	CONCENTRATION ADDED	AVERAGE CONCENTRATION FOUND	AVERAGE RETENTION	AVERAGE LOSS
	p.p.m.	*p.p.m.*	*per cent*	*per cent*
Methoxychlor	2	0.132	6.60	93.40
	5	0.181	3.62	96.38
	10	0.438	4.38	95.62
	Average		4.87	95.13
DDD	Average		48.8	51.2
Parathion	0.01	0.004_8	48	52
	0.02	0.008_6	43	57
	0.05	0.018_9	37.8	62.2
	0.1	0.039_3	39.3	60.7
	0.2	0.071_1	35.6	64.4
	0.5	0.185_1	37.0	63
	1.0	0.373_7	37.4	62.6
	2.0	0.759_0	38.0	62
	5.0	1.81	36.2	63.8
	10.0	3.34	33.4	66.6
	Average		36.8	63.2

complete extraction of the residue from the product, considerable effort was made to effect as complete an extraction as possible.

To determine the efficiency of the extraction a known weight of insecticide was blended for two minutes with a processed product. The insecticide was then immediately extracted and its quantity determined. The efficiency of the extraction was determined for each product at each concentration and a correction was made for the unextracted residue.

The extraction procedure was essentially that of Tressler (4) using benzene as the solvent. One important modification was made; the product and solvent were blended together for at least five minutes before any sodium sulfate was added. This technique greatly increased both the quantity and uniformity of the yield. Parathion was the most difficult to extract of the insecticides studied, but by the modification described above it was possible to effect a removal of about 85% of the parathion

present, with a minimum value of 78% and a maximum of 97%.

It should be pointed out that below 0.05 p.p.m. the error in the determination of parathion residues is so great that the results of the analyses at the 0.01 and 0.02 p.p.m. levels are of little value.

DISCUSSION OF RESULTS

In any evaluation of the results of these analyses, it must be borne in mind that the analytical procedures employed are not necessarily specific for the molecule under consideration and that small changes in the structure of the insecticide are possible without being evidenced in the results. On the other hand, the methods are, in general, sufficiently specific so that any significant change in the structure would be detected. It will be noticed that for any given insecticide there is a general agreement in the percentage of breakdown for all products studied and at all concentrations, except for parathion when this does not hold true at very low concentrations, where the experimental errors in the analytical method are extremely high, and for the isolated case of parathion in peaches. It is interesting to note that the final processing of the product has but little effect upon the parathion breakdown as is shown in the study of retorted and non-retorted peas. It is evident here that the breakdown encountered occurred during the preliminary cooking.

TABLE 6.—*Average loss, for concentration 0.05 to 10.0 p.p.m., of added parathion on processing*

PRODUCT	RETENTION	LOSS
	per cent	*per cent*
Applesauce	34.8	65.2
Apricots	46.1	53.9
Peaches	65.1	34.9
Pears	26.4	73.6
Peas—Not retorted	35.4	64.6
Peas—Retorted	30.1*	69.9*
Spinach	26.2	73.8
Vegetable & Beef Soup	25.4	74.6

* Average of concentrations 0.1 to 10.0 p.p.m.

In Tables 5 and 6 these data on the loss of insecticides following processing are summarized. It will be noted that in Table 5 the average for all products of the percentage of parathion broken down is relatively uniform for initial concentrations between 0.05 and 10 p.p.m. with a decrease in the average loss at 0.01 and 0.02 p.p.m. We feel that the data are too unreliable to attach any significance to this last point. On the other hand, there is a variation from 74.6% to 34.9% loss when the average of all concentrations of parathion of 0.05 p.p.m. and above in a single product are compared (Table 6). However, only one product, peaches, differs

greatly from the average. Considerable work has been done in an attempt to determine the cause of these unusual figures for peaches but no satisfactory explanation has been found. These abnormal results may be due entirely to experimental error, but the source of such an error is not obvious.

We would like to bring out one additional point in connection with the data on parathion. The results of these analyses do not show the amount of undestroyed parathion in the product but rather the amount in the solvent extract. From numerous experiments over the past year or so we have collected some evidence that parathion is either absorbed or adsorbed by some constituent of the food product and is not entirely released by the extraction method used. This sorption may be increased by cooking so that the loss shown above is only apparent. It is questionable that such is actually the case, but the possibility should not be overlooked.

The study of the development of off-flavors in products containing the 98% gamma isomer of benzene hexachloride indicates that these off-flavors develop over a period of several weeks after processing and then drop off after a year. Other data not presented in this report confirm these findings. It should be remembered that with anything as subjective as taste testing it is difficult to translate the results into statistical data. In Tables 3 and 4 an attempt has been made to do this. The results are expressed as the fraction (to one decimal place) of the total number of tasters finding any off-flavor. This fractional method was used in preference to percentages to emphasize the fact that the data are not precise and therefore not capable of being expressed to two significant figures. Furthermore, these figures do not show the degree of off-flavor, such as a vaguely detectable BHC flavor compared with a strong penetrating BHC taste.

A study is now under way to determine the nature of the breakdown products resulting from processing an insecticide but as yet we have no definite facts to report. We have found that when 98% gamma isomer benzene hexachloride is added to water containing small amounts of various reagents and the resulting solutions processed at 240°F. for 30 minutes that odors and flavors are developed which resemble those of technical benzene hexachloride. A spectrophotometric examination of an ether extract of such solutions has failed to show any ultraviolet absorption peaks corresponding to trichlorobenzene, the most common breakdown product of benzene hexachloride.

SUMMARY

It has been shown that parathion, methoxychlor, and DDD break down to various extents upon processing with average losses of 63.2% for parathion, 95.1% for methoxychlor, and 51.2% for DDD. Furthermore it has been shown that the 98% gamma isomer of benzene hexachloride will develop a flavor, after processing, resembling that of the crude product.

Further experiments are under way along these same lines of investigation using a larger variety of insecticides, and an attempt is being made to isolate and determine the nature of various breakdown products.

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REPORT ON COPPER IN FOODS

By W. C. STAMMER (Continental Can Company, Inc., Chicago, Illinois), *Associate Referee*

Present A.O.A.C. methods for the determination of copper in foods, namely, the volumetric method **29.00** and the diethyldithiocarbamate method **29.14**, are classified as tentative (12). These procedures are rather insensitive and therefore require large samples if the analyst is interested in micro amounts of copper. Large samples are difficult to handle in wet and dry ashing preparations where a great number of samples must be run.

Wichmann, Greenleaf, and others (8), (4) prior to 1944, recognized the need for a sensitive micro method for determining copper in foods. In 1944 Wichmann (9) called attention to the method of Bendix and Grabenstetter (3) and recommended that the all-dithizone method be studied after perfection of the carbamate method. Considerable work had gone into the carbamate procedure and this method was completed by Greenleaf (5) (7) and recommended as tentative in 1945 and later published in the 6th Edition of the A.O.A.C. *Methods of Analysis.*

After the introduction of the single color method of Bendix, certain criticisms of the method were put forth. Preliminary extraction of the copper at *p*H 2.3 for 10 minutes required a mechanical shaker for a thorough extraction. Later work indicated that an extraction time of four minutes at a *p*H of 3.2 would be adequate and shaking could be done by hand.

Concern was also expressed over the stripping of the excess dithizone with dilute ammonium hydroxide because of the possibility of dissociation or enolization of the copper dithizone complex resulting in low copper values.

In 1947 Greenleaf (6, 7, 11) published a mixed color method overcoming some of the theoretical deficiencies attributed the Bendix method.

Bendix (1) as Associate Referee and at the recommendation of Wichmann in 1947, conducted comparative tests on the one and two color

methods and listed the advantages and disadvantages of each. In 1948 the Greenleaf and Bendix procedures were submitted to collaborative study. (2) The results of this study indicated that the Greenleaf method was rather tedious and time consuming while the Bendix method produced acceptable results with a minimum of effort. The Associate Referee recommended that in instances where a more theoretically perfect method is required, the Greenleaf method be accepted as a referee method pending official adoption, and for a routine procedure the Bendix method be accepted pending official adoption. It was felt that further collaborative study would reconcile some of the difficulties encountered and place these procedures on sound basis for official adoption.

COLLABORATIVE STUDY

Three foods samples were chosen for this study, namely dried corn, starch, and tomato juice. The samples were selected to provide copper levels of 0–5, 5–10, and 10–15 p.p.m. copper. The samples were forwarded to six collaborators along with instructions that they determine copper by the Greenleaf and Bendix-Grabenstetter methods.

The results of this study are incomplete. Several of the collaborators were unable to finish their work in time to be included in a report to this meeting. The Associate Referee will urge these collaborators to make every effort to complete their work so that the results of the study can be presented at a later date.

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REPORT ON SODIUM FLUOROACETATE (1080)

By L. L. RAMSEY, *Associate Referee*, and P. A. CLIFFORD (Division of Food, Food and Drug Administration, Federal Security Agency, Washington, D. C.)

A paper entitled "Determination of Monofluoroacetic Acid in Foods and Biological Materials" was published in November, 1949.[1] The details

[1] *This Journal*, **32**, 788 (1949).

of this method will also appear in the 7th Ed. *Methods of Analysis*, 1950.

The method was subjected to collaborative study. Two series of samples were prepared with each of two foods, sugar and flour; to one series 1.76 p.p.m. of monofluoroacetic acid was added as the potassium salt in aqueous solution and the sample allowed to dry; and to the other series, 8.80 p.p.m. was likewise added. Samples 1, 2, 3, and 4 were to be analyzed and if the analyst had time also samples D, C, B, and A, respective duplicates, such fact, however, being unknown to the collaborators. The collaborators were supplied with the following reagents: a solution of pure potassium

TABLE 1.—*Collaborative results*

	SUGAR		SUGAR		FLOUR		FLOUR	
ADDED, FLUOROACETIC ACID	1	D	3	B	4	A	2	C
	1.76	1.76	8.80	8.80	1.76	1.76	8.80	8.80
Found:								
Analyst 1, p.p.m.	1.7	—	8.3	—	—	1.9	—	7.6
Analyst 2, p.p.m.	1.3	1.6	8.1	8.3	1.6	2.2	8.1	8.4
Analyst 3, p.p.m.	2.1	—	8.0	—	1.9	—	7.9	—
Analyst 4, p.p.m.	1.4	—	7.3	—	1.5	—	8.0	—
Analyst 5 & 6, p.p.m.	0.8	1.2	7.6	8.0	0.8	1.0	6.1	6.6
Analyst 7, p.p.m.	—	—	—	—	—	—	8.4	—

monofluoroacetate; standard solution of potassium fluosilicate; tertiary amyl alcohol; and lime suspension. The collaborators were specifically instructed to use the entire 100 g. sample for the determination in each case and to use 3.5 ml. of ca 0.5*N* H_2SO_4 with 5 g. of the silicic acid they received, unless the silicic acid agglomerated in the solvent, in which case the 0.5 *N* H_2SO_4 was to be reduced by 0.1 to 0.3 ml. Otherwise the procedure is exactly like that in the published method. The collaborators were also instructed to obtain a recovery on 1 mg. of fluoroacetic acid (2 ml. of the pure salt solution which was sent them), carrying the determination through the entire prodecure beginning with the extraction step. They were requested to report the percentage recovery obtained on the solution of pure acid (known) and to report the p.p.m. of fluoroacetic acid found in the collaborative food samples (unknowns).

Recoveries from an aqueous solution of the pure compound (1 mg. fluoroacetic acid added) are very good, 93, 92, 91, 87, 94, and 94% for analysts 1 to 6, respectively, considering the quantity of fluoroacetic acid involved and the nature of the determination.

Recoveries from the food samples are somewhat lower on the average (Table 1); at the lower level (1.76 p.p.m.) they range from 0.8 to 2.2 p.p.m., and at the higher level (8.80 p.p.m.) from 6.1 to 8.4 p.p.m.

The results of one collaborator are not included in the table because it

was not possible for him to follow the method exactly, the reason being lack of adequate equipment.

RECOMMENDATIONS*

(1) It is recommended that this method (*This Journal*, Nov. 1949) for the determination of 1080 in foods be adopted, first action.

(2) It is further recommended that study of the method be continued for the purpose of placing it on a more quantitative basis.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 54 (1950).

TUESDAY—AFTERNOON SESSION

REPORT ON VITAMINS

By CHESTER D. TOLLE (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Referee*

The reports given this year are adequate evidence of the diligence of the Associate Referees and of the continued interest and best of cooperation among members of this Association in meeting the problems of application of vitamin assay methods to complex biological materials.

RECOMMENDATIONS*

The Associate Referee on Vitamin A has reported on an extensive collaborative study, carried out under auspices of the U.S.P. Vitamin Advisory Board, of a spectrophotometric method for vitamin A. Although a physico-chemical method for vitamin A in fish liver oils was adopted as official, first action, last year, there is now adequate basis for inclusion of a calculation based on the Morton-Stubbs correction technic. I concur in J. B. Wilkie's recommendation which is as follows:

It is recommended that the spectrophotometric method for vitamin A in fish liver oils as adopted last year be modified to include the use of the U.S.P. Reference Standard for vitamin A and the Morton-Stubbs correction.

An important contribution this year is the extensive study of methods for measuring vitamin A in mixed feeds. The newly appointed Associate Referee on this subject has been successful in bringing together a number of similar procedures that have been in use for this purpose and in formulating a single method that has been evaluated in a preliminary collaborative study. Although results of this study are considered insufficient as a basis for approval of a specific method this year, they point the way for further work. I concur in the recommendation of H. C. Shaefer to continue study of this subject during the coming year.

The determination of carotene in feedstuffs has been under study by this Association for a number of years and many difficult problems have been dealt with. The Associate Referee this year has attempted, by an extensive survey of workers in this field, to define the most important remaining sources of error. He has recommended important modifications of the procedure and has given much thought to the direction that additional study shall take. I concur in recommendations of F. W. Quakenbush, as given in his report on Carotene.

Although no new collaborative work on vitamin D for poultry has been done, a desirable change in the Reference Standard is now possible. This entails a change in the designation of the chick unit, as well. I concur

* For report of Subcommittee A and action of the Association, see *This Journal*, 33, 43 (1950).

in Dr. Friedman's recommendations in this regard, and approve of his suggestions for further work an vitamin D in poultry feed supplement.

Some of the reports presented this year are concerned mainly with changes in designation of previously adopted methods to first action or official status in accordance with the request of the Committee on Classification of Methods. This is true of the reports on thiamine, riboflavin, and niacin. Each of these contains recommendations for minor modifications or editorial change or correction as well. I concur in the following:

Dr. Kline's recommendations as given in his reports on thiamine and nicotinic acid.

Dr. Loy's recommendations in his reports on the microbiological method and the fluorometric method for the assay of riboflavin, with editorial changes and modifications.

With respect to the sublimation method for the determination of nicotinic acid, it is recommended that the procedure appearing on p. 620 in Sixth Edition of *Methods of Analysis* be deleted. This method involves the process of sublimation and, although applicable to relatively pure solutions and tablets of nicotinic acid, it is lacking in specificity. Methods involving cyanogen-bromide and an aromatic amine are now in wide use and appear satisfactory for several types of materials. I recommend appointment of an Associate Referee on this subject with instructions to study such methods during the coming year.

As a result of her searching investigation reported last year the Associate Referee on Folic Acid was faced with choosing a single microorganism as the test subject. The collaborative work conducted this year serves as adequate basis for selecting *S. faecalis*, and demonstrates the general suitability of the method recommended. Important problems of extraction of the vitamin remain to be solved and must be the subject of future study. I concur in Miss Flynn's recommendation that the microbiological assay method for folic acid subjected to collaborative assays in 1948 and 1949, using *S. faecalis* as the test organism, be adopted as first action.

Vitamin B_{12} and the animal protein factor appear to be important in animal feeding and concentrates of them are coming into wide use. It is important that prompt efforts be made to describe suitable methods for their determination in such concentrates and in feeds. I recommend appointment of an Associate Referee on this subject.

REPORT ON VITAMIN A

By J. B. WILKIE (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

During the past year two developments have occurred that make it possible to recommend important changes in the A.O.A.C. method for the spectrophotometric determination of vitamin A, that appears in 6th edition of *Methods of Analysis* as tentative.

In April of this year, at a conference of experts on fat-soluble vitamins, a sub-committee of the Committee on Biological Standardization of the World Health Organization, recommended a new International Standard for Vitamin A. This recommendation and adoption by the World Health Organization will be published in the Chronicle of the World Health Organization. It was recommended that the International unit shall be the activity of .344 μgm. of the standard crystalline vitamin A acetate, which is the equivalent of .3 μgm. of vitamin A alcohol. This is consistent with the present U.S.P. Standard. The previous international standard for vitamin A, which was a solution of B-carotene in oil, is being retained as a standard for pro-vitamin A.

The adoption of the new standard will have the effect of settling a dispute of long standing on the conversion factor for E value to biological unit. The conversion factor is now fixed by definition and may be calculated in the following manner.

For the Alcohol:

By definition

$$.3\ \mu\text{gm.} = 1 \text{ unit or } 1 \text{ gram} = 3{,}333{,}000 \text{ units}$$

$$1750 = E^{1\%}_{1\text{cm.}}\ 325\ \text{m}\mu \text{ experimental value}$$

$$\text{Then } \frac{3{,}330{,}000}{1750} = 1900 \text{ conversion factor}$$

For the Acetate:

Following from the definition

$$.344 \text{ gm.} = 1 \text{ unit}$$

or

$$1 \text{ gram} = 2{,}920{,}000 \text{ units}$$

$$1525 = E^{1\%}_{1\text{cm.}}\ 325 \text{ experimental value}$$

$$\text{Then } \frac{2{,}920{,}000}{1525} = 1900 \text{ conversion factor}$$

It is especially interesting to note that the International Subcommittee recognized the need for stating criteria of applicability of the method of obtaining spectrophotometric data. They set forth the following conditions:

(a) That the absorption maximum shall be within the range 325–328 mμ.

(b) That the shape of the absorption curve in the region 310–350 mμ

shall be in agreement with the absorption curves for the A alcohol and the acetate. Specifically, intensities of absorption between the unknown and the standard in the region 310–350 mμ expressed in decimal fractions of the maximum should not differ by more than .02.

It is also significant that the International Subcommittee considered saponification as an important step in the procedure. They further recommended the use of a Morton and Stubbs correction for those oils beyond the limits specified above, or where saponification fails to provide a curve within the required limits.

A second important development during the past year was the report of an extensive collaborative study of a spectrophotometric method for vitamin A conducted under the guidance of an informal committee, given this assignment by the Vitamin Advisory Board of the U.S.P. Twenty-three laboratories took part and followed a proposed spectrophotometric method, which included a correction calculated according to the Morton and Stubbs method. Also studied was a proposed antimony trichloride procedure included for comparative purposes. The details and results of this study will be found elsewhere. Your Associate Referee served as a member of the informal committee and, in his opinion, results of the study are a satisfactory basis for certain recommendations for change of the present A.O.A.C. method. The major modifications concern the use of the Morton and Stubbs correction and the addition of the antimony trichloride method used for checking the validity of the spectrophotometric value. The complete procedure is too long to present at this time. However, mention of the more salient points may clarify the situation.

The method, as proposed, would pertain only to fish liver oils. It should, however, serve as a foundation of other vitamin A-containing materials. The spectrophotometric method proper, including the saponification and extraction steps, does not depart appreciably from the method now in the 6th edition, *Methods of Analysis*. However, density wave length readings are taken at the "fixation" points of 310 mμ, 325 mμ, and 334 mμ. These readings are for substitution in a U.S.P. committee simplified formula for applying the Morton and Stubbs correction. The formula will provide results directly in micrograms per unit of sample, rather than in the usual U.S.P. biological units per gram. The factor of 3.34 is used for conversion from micrograms to biological U.S.P. units.

The gravimetric method of expressing results was chosen in place of the U.S.P. biological units used previously because it is a more definite expression of spectrophotometric results, and it avoids the implications of biological assays.

The method of calculation avoids the use of $E^{1\%}_{1cm.}$ values entirely and uses a formula which facilitates the use of both gram as well as other than gram sampling units.

The use of the antimony trichloride test, as an identity or validity test,

for the spectrophotometric method with the Morton's correction is an innovation of the U.S.P. committee. It is based upon many years' experience but perhaps more largely upon the more recent extensive collaborative studies undertaken by this committee. To indicate proper identity or validity, the U.S.P. committee has stated that the antimony trichloride values must be between 1 and 1.3 times the Morton corrected spectrophotometric valuse. To the purist, familiar with the derivation of Morton and Stubbs correction, such a test may appear unnecessarily empirical. However, empirical or not, it has a demonstrated practicality and usefulness. It is for this reason that I recommend* its approval with only minor revisions.

However, in this connection it appears that this method of testing validity may possibly be superseded, or possibly just supplemented, by the results of further study to arrive at a reasonably simple application of Morton's own criteria of applicability. More specifically, it appears that such a criteria already exists in a dormant manner in the three fixation points as are now required in the U.S.P. modified Morton's correction. In fact, a formula for such use has been derived but there has been no opportunity to adequately test it.

RECOMMENDATION*

In view of the above considerations it is recommended that the physical-chemical method for vitamin A be adopted as "first action." Details of the method are published in *Methods of Analysis*, 7th Ed. (1950).

REPORT ON VITAMIN A IN ANIMAL FOODS

By H. C. SCHAEFER (Ralston Purina Co., St. Louis, Mo.),
Associate Referee

The widespread use of vitamin A from fish oils or other concentrated sources in present day mixed feeds has brought about a need for a satisfactory standard procedure for the measurement of vitamin A in such products.

Several methods applying to measurement of vitamin A in mixed feeds have been published. Chronologically these include the methods of Cooley, Christiansen, and Schroeder (1), Brew and Scott (2), Narod and Verhagen (3), Wall and Kelley (4), and a revised method by Cooley, Christiansen, and Koehn (5), which had not been published at the time this work was started.

A questionnaire sent to all prospective collaborators asking for suggestions as to the method to be studied produced two additional detailed procedures by Quackenbush, Hauge, and Burns (6) and by Thompson (7) respectively. The revised procedure by Cooley, Christiansen, and

* For report of Subcommittee A and action of the Association, see *This Journal*, 33, 43 (1950).

Koehn was kindly furnished while still in press. In addition numerous suggestions as to details of procedure were forthcoming from the collaborators.

An examination of available procedures, together with the suggestions offered on the questionnaires, revealed many details common to most suggested methods. An attempt was then made to incorporate into a workable analytical method those details of procedure concerning which there was most general agreement. When such a method had been evolved a number of experiments were conducted to check upon the workability of the method as compared to literature procedures. The method finally selected is probably most noteworthy for the fact that it is in no way original.

COLLABORATIVE METHOD

The following method and general precautions were given to all collaborators who had offered to participate.

GENERAL PRECAUTIONS

1. Run all procedures in subdued artificial light if possible. Avoid direct sunlight or other strong light.
2. Use non-actinic glassware if available.
3. Use vacuum with steam or hot water heat for all evaporations. Refill flask after evaporation with nitrogen if practical. A T tube and balloon may be used for this purpose.
4. Make color readings as rapidly as possible taking maximum density value attained.
5. Store all samples in a cool, dark location.
6. Run samples as soon as possible after they are received. Report date for each assay.

REAGENTS

(1) *Hexane.*—Normal hexane or Skellysolve "B".

(2) *Acetone.*—Reagent grade.

(3) *Magnesia.*—Adsorptive powdered magnesia No. 2641 from Westvaco Chlorine Products Corp., Newark, California.

(4) *Super Cel.*—Hyflo Super Cel, Johns-Manville Co.

(5) *Sodium sulfate.*—Anhydrous reagent grade.

(6) *Chloroform.*—Reagent grade containing 0.5% ethanol as a preservative.

(7) *Antimony trichloride.*—Reagent grade free from dark colored impurities.

(8) *Acetic anhydride.*—Reagent grade.

(9) *Extraction soln.*—30% by volume acetone in hexane.

(10) *Adsorbent.*—Equal parts by weight of magnesia and Super Cel.

(11) *Elution soln.*—7% by volume of acetone in hexane.

(12) *Antimony trichloride soln.*—Dissolve 90 g of antimony trichloride in 240 ml of chloroform by heating on a steam bath. Let the soln stand for 24 hours, filter and keep in the dark. Wide-mouth glass stoppered bottles are convenient for this purpose.

(13) *Vitamin A standard.*—U.S.P. Vitamin A Standard capsules obtainable from the United States Pharmacopoeia Reference Standards, 4738 Kingsessing Ave., Philadelphia 43, Pa. Each capsule contains 2500 U.S.P. units of vitamin A at a concentration of 10,000 U.S.P. units per g. For use as an internal standard dis-

solve the contents of a capsule in hexane to give a concentration of 10 U.S.P. units per ml or other suitable concentration depending upon the instrument used. For use in obtaining a reference curve, dissolve in chloroform and make suitable dilutions.

EXTRACTION OF MIXED FEEDS

Reflux a 10-g sample of the mixed feed for 60 min. with exactly 100 ml of 30% acetone in hexane soln, preferably in an all glass flask and condenser set up, providing heat by means of a steam or water bath. (The apparatus should be set up with sufficient condensation provision to avoid volume changes.) After refluxing, cool, allow to settle and pipette off a 50-ml aliquot into a flask suitable for vacuum evaporation. Evaporate under a vacuum to a volume not exceeding 5 ml. Add 25 ml of hexane and the sample is ready for chromatographing.

CHROMATOGRAPHY

Column.—Prepare a column, ca 18–20 mm inside diameter, by first adding a 1:1 mixture of magnesia and Super Cel to a height of 15 cm and then applying vacuum. Press the adsorbent surface down with a suitable plunger with vacuum applied. The final depth of packed volume should be about 10 cm. Add a 1–2 cm layer of anhydrous sodium sulfate to the top of the column.

Adsorption and elution.—Add the extract in hexane to the column, wash out the flask with a small amount of the 7% elution soln and add to the column just as the last of the original extract passes into the column. Note that the column should never be allowed to run completely dry. Follow the small wash with more 7% elution soln added to the column until the vitamin A, but not any carotene, has passed thru the column.

The passage of the vitamin A can be followed on most feed extracts containing any carotene by the fact that vitamin A ester will precede the first yellow zone produced by the carotene. Elution should be continued until the eluate just starts to show a yellow tinge from the carotene. It is important that only a trace of carotene be permitted to enter the vitamin A solution.

If only small amounts of carotene are present, the passage of vitamin A may be followed using ultra-violet light. This light should only be used for momentary examination of the column. The vitamin A ester can be detected as a greenish yellow band proceeding down the column ahead of all other bands.

When elution of vitamin A ester is complete, the carotene can be removed by continued elution into a second receiver and measured by the usually recommended procedures.

In order to compare results by an internal standard procedure with these obtained from a calibration curve, the eluate is split into two equal volumes at this stage.

To one portion, add 10 U.S.P. units of vitamin A in hexane (or other suitable amount depending upon type of instrument used) for each ml. of chloroform to be used in final dilution. This will provide an internal standard of 10 U.S.P. units per ml. for reaction.

The fortified and unfortified portions of the eluate are evaporated to dry-

ness under a vacuum and an equal volume of chloroform added to each. The volume of chloroform to use depends upon the original potency of the material and the approximate potency desired for reading. The chloroform volume may be estimated as follows to give an approximate final potency of 15 and 25 U.S.P. units per ml. for the unfortified and fortified samples respectively.

$$\text{Chloroform volume (each fraction)} = \frac{\text{estimated vit. A units per g. feed}}{6}$$

This should be estimated to the nearest whole number. On original potencies of less than 9 U.S.P. units/g. the final part of the evaporation procedure should be carried out in the colorimeter tubes to be used for reading and 1 ml of chloroform added to both unfortified and fortified portions in the colorimeter tubes.

COLOR REACTION

A 1-ml. portion of both the unfortified and fortified chloroform solutions are used for the vitamin A reaction.

The instrument (this description fits an Evelyn colorimeter, directions and volumes may have to be altered for other colorimeters) is set for a wave band with peak at 620 mμ. With the instrument turned on, a tube containing 1 ml of chloroform plus one drop acetic anhydride is placed in position for reading. Ten ml of antimony trichloride solution are quickly added from a fast emptying pipette and the instrument set for a blank value of 100% transmission. The blank solution may be removed and without an adsorption tube in place a reference reading taken for setting the instrument for a series of samples. This avoids the necessity of repeating the blank reading for each sample.

The fortified and unfortified portions of the sample are then read on 1 ml aliquots using one drop of acetic anhydride in each, added just before reaction.

Transmission readings are then converted to corresponding photometric densities and calculations carried out as follows:

1. Reference curve procedure.—The vitamin A concentration of the unfortified sample is looked up on a previously prepared standard curve based on standard vitamin A solutions reacted under conditions identical with that of the samples. Calculate vitamin A from the formula:

$$\text{U.S.P. units per g of feed} = \frac{(\text{Units in aliquot})(\text{Chloroform vol.})}{2.5}$$

In which

Units in aliquot = Reading on 1 ml of unfortified sample converted to vitamin A units by means of the reference curve.

Chloroform volume = Total volume of chloroform added to make up the unfortified fraction.

2. Internal standard procedure.

$$\text{U.S.P. units per g of feed} = \frac{10(A)(\text{Chloroform vol.})}{(B-A)(2.5)}$$

In which

A = photometric density given by 1 ml aliquot of unfortified fraction.

B = photometric density given by 1 ml aliquot of fortified fraction (10 units per ml).

Chloroform volume = Total volume of chloroform added to make each fraction.

SAMPLES

Samples of the composition given in Table 1 were submitted to all collaborators participating. The composition and approximate calculated vitamin A content of all samples except No. 6 were given to the collaborators. Sample No. 6 was a commercial mixed feed formula which had been fortified to the calculated vitamin A level shown in the table. The composition and calculated vitamin A content of sample No. 6 were not given to the collaborators.

TABLE 1.—*Composition of experimental feed mixtures*

SAMPLE NO.	1	2	3	4	5	6
Corn, per cent	50	50	50	50	50	
Soybean Oil Meal, per cent	35	35	15	15	15	
Dehydrated Alfalfa, per cent	—	15	—	15	15	Commercial
Fish Meal, per cent	—	—	10	10	10	Mixed Feed
Meat Scrap, per cent	—	—	10	10	10	
Ground Wheat, per cent	15	—	15	—	—	
Calculated vitamin A (Approx. U.S.P. Units per g)	18	18	18	18	0	12

DISCUSSION OF METHOD, SAMPLES, AND PRECAUTIONS

A discussion of the method, samples, and general precautions to be used in the collaborative work was sent to all collaborators at the time the method was submitted.

A wide variety of extraction procedures had been suggested and the evidence for selection was somewhat conflicting. The method finally selected was similar to one suggested by several collaborators and was chosen because it was essentially the same as the method used for carotene extraction in the new A.O.A.C. procedure. Certain advantages to such selection, especially when carotene as well as vitamin A was to be measured on a mixed feed, were obvious. Tests run by several collaborators as well as the laboratory of the associate referee had indicated that this method was as good as any other proposed. Conflicting data however made impossible complete justification of any single extraction procedure. This point will probably provide a variable for future collaborative work.

In applying any extraction procedure it is well to remember that certain devices employed for the protection of the vitamin A present may make any particular method inapplicable. A specialized knowledge of the particular sample is required in such cases.

Omission of a saponification step was in agreement with the large majority of collaborators. The advantages in simplification of procedure and in reduction of vitamin A losses in process are quite evident. The greatest disadvantage to such omission is the difficulty of applying the method to both the ester and alcohol form of vitamin A. On the basis of our knowledge of the presently used sources of vitamin A plus the fact that the greater stability of the ester indicates such a form for any future synthetic products, it appeared that a method useful only in measuring the vitamin A esters would be sufficient. The collaborative method was therefore set up on such a basis.

Magnesium oxide is widely recommended for separation of vitamin A esters from carotene and other plant pigments. For the sake of uniformity and on the basis of considerable experimental evidence the brand recommended in the procedure was chosen. This adsorbent differs only slightly from that chosen for carotene purification in the A.O.A.C. procedure but the slight difference appears to favor better separation of vitamin A ester and carotene.

Slight variations in column size, depth of packing, etc., should not materially affect the results. The ratio of magnesia to Super Cel was that recommended by several collaborators. Other proportions were recommended and might be justified. Variation of these proportions would alter somewhat the size column required and the acetone concentration required for elution.

Samples containing any carotene can be followed on the column by means of the carotene band which comes thru the column just after the vitamin A ester. Ultra violet light has been recommended and is useful, particularly when little or no carotene is present. Users of the method were referred to the references given for details of the use of ultra violet light for this purpose.

The color forming reagent almost universally used is antimony trichloride. Several collaborators suggested the use of glycerol dichlorohydrin but few of these indicate any experience with this reagent. In view of the wider experience of the collaborators with antimony trichloride, plus the fact of its greater sensitivity and some uncertainties connected with the activation of the glycerol dichlorohydrin reagent, it seemed wise to select antimony trichloride for use.

Reading techniques differ somewhat between various recommended procedures. The technique described was set up for study in such a way that both a calibration curve or an internal standard method of reading could be used. This enabled a comparison of these two methods to be

made. It is important that a minimum amount of carotene be allowed to elute into the vitamin A fraction and this should be watched quite closely. The carotene fraction if present in the vitamin A eluate will result in high values.

The use of recovery data on duplicate parallel samples with known additions of vitamin A to provide a correction factor was suggested and had much to commend it. This would of course serve the purpose of an internal standard in the reading procedure. Its use is predicated upon the assumption that all losses in parallel assays are proportional to the amount of vitamin A present. Evidence was available to indicate that some losses may be absolute rather than proportional. It appeared undesirable to double the work required for the method unless this later appeared as an absolute necessity.

In order to secure information from this study which could be interpreted as accurately as possible several variations from practical mixed feed formulas were considered desirable.

First, the vitamin A in all of the collaborative samples was set at a level which was somewhat above that required in many types of mixed feeds. This was done so as to provide a vitamin A value sufficiently high to enable interpretation of the variation in the results.

Second, the formulas used were greatly simplified and not strictly representative of mixed feed practice. They were set up largely for the purpose of providing results which could be interpreted in terms of a general class of materials which might cause interference with the method. The method of adding the vitamin A and the formulas used were both designed to minimize vitamin A losses before analysis and yet not to have the vitamin A in any form difficult to extract. In the formulas given the limitations and weaknesses of the method could best be discovered.

The formulas and the approximate vitamin A content of five of the six samples were given. Number six was a completely blind sample introduced as a statistical control. The use of known formulas it was hoped, would encourage further research on the part of collaborators and enable them to perceive the effect of different formula variables upon the procedure. It was particularly emphasized that duplicate samples should be run on different days and all values reported except in cases of known departure from the procedure. It was to be expected that on some of the samples at least, wide variation from the nominal values and between duplicates would be found. From the standpoint of interpreting the limitations and utility of the method, such variations were of equal importance with data agreeing well with expected values.

All collaborators were encouraged to use any other method with which they might be familiar or to report on any variation of the given method in addition to the data requested. Details of any other method or variations were requested.

In order to minimize storage effects, the accompanying method was sent one month ahead of the samples. Collaborators were urged to familiarize themselves with the details of the method, and to provide the necessary supplies in the interim so that the actual samples could be handled as promptly as possible. Excessive delay in analysis would affect the results and space was provided in the report form for the date of analysis in every case. Samples when received were to be stored in a cool, dark place.

It was emphasized that all collaborators should use the same vitamin A unit and standard. The directions on the standard in the accompanying method were to be closely followed. It was understood that the unit recommended for this work did not agree with some commercial practices.

It was recommended that the precautions of low light intensity, low actinic glassware, the use of inert gases in evaporation procedures etc. should be followed when practical. Absence of direct sunlight and the use of vacuum for evaporation were indicated as essential.

Details of instrumental technique in reading were left to the individual laboratories. The method as outlined applied to an Evelyn type colorimeter. Variation in the reading procedure may be required to suit other makes of instruments. Space was provided in the report for details of such variation.

LIST OF COLLABORATORS

R. E. Anderson, Archer Daniels Midland Co., Minneapolis, Minn.
L. H. Almy, H. J. Heinz Co., Pittsburgh, Pa.
C. E. Calverly, Russell Miller Milling Co., Minneapolis, Minn.
M. E. Christensen, State Chemist, Salt Lake City, Utah
M. L. Cooley, Larrowe Div., General Mills, Inc., Rossford, Ohio
W. R. Flach and C. D. Sanders, Eastern States Farmers Exchange, Buffalo, N. Y.
S. H. Fox, Gelatin Products Div., R. P. Scherer Corp., Detroit, Mich.
P. R. Frey, Colorado A & M College, Fort Collins, Colo.
H. H. Hoffman, Florida Dept. of Agriculture, Tallahassee, Fla.
S. R. Johnson and M. P. Fallin, M. F. A., Milling Co., Springfield, Mo.
C. H. Krieger, R. Nell, and W. D. Lewis, Wisconsin Alumni Research Foundation, Madison, Wis.
J. W. Kuzmeski, Agr. Exp. Sta., Massachusetts State College, Amherst, Mass.
D. J. Mitchell, South Dakota State Chem. Lab., Vermillion, S. Dak.
J. H. Mitchell, South Carolina Exp. Sta., Clemson, S. C.
K. Morgareidge, Nopco Chemical Co., Harrison, N. J.
M. Narod, Lyle Branchflower Co., Seattle, Wash.
B. L. Oser, Food Research Laboratories, Long Island City, N. Y.
F. W. Quackenbush and M. J. Burns, Agr. Exp. Sta., Purdue University, Lafayette, Ind.
F. E. Randall, Cooperative G. L. F. Exchange, Buffalo, N. Y.
L. Rosner, H. Kan, and G. Siegel, Laboratory of Vitamin Technology, Chicago, Ill.
H. C. Schaefer, W. Brew, and A. Schulz, Ralston Purina Co., St. Louis, Mo.
C. K. Shuman, The Glidden Co., Indianapolis, Ind.

H. K. Steele, The Fleischmann Laboratories, Standard Brands Inc., New York, N. Y.
W. S. Thompson and D. M. Stalter, State of Ohio Dept. of Agr., Columbus, Ohio.
M. E. Wall, Eastern Regional Research Lab., U. S. D. A., Wyndmoor, Pa.
E. M. Wilkie, Food and Drug Administration, Washington, D. C.

TABLE 2.—*Average values found for vitamin A in collaborative samples*
Vitamin A values calculated from standard curve

COLLABORATOR	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6
1	17.0	16.8	17.0	16.5	1.1	11.3
2	12.4	13.9	14.4	14.6	1.0	11.0
3	10.8	10.7	11.8	9.1	0.3	6.8
4	15.2	13.2	14.1	13.7	0.5	9.4
5	19.7	20.0	20.4	15.5	1.7	10.7
6	11.9	12.8	14.1	13.0	0.7	8.4
7	15.5	16.2	14.8	15.8	1.0	11.3
8	17.1	14.2	12.9	13.2	—	—
9	17.3	16.6	16.4	17.0	0.4	12.1
10	13.2	12.3	12.7	13.8	0.7	7.5
11	15.0	12.4	14.6	15.9	0.4	10.6
12	—	—	—	—	—	—
13	18.5	20.2	18.8	18.6	0	11.5
14	9.0	19.1	15.4	12.6	3.0	6.8
15	16.8	14.4	13.9	14.2	0.9	9.3
16	14.1	16.2	16.3	16.8	1.0	11.9
17	18.8	14.9	18.6	15.4	0.6	9.5
18	14.1	12.0	17.4	12.9	0.8	10.4
19	14.6	12.8	17.4	13.4	0.8	7.3
20	—	—	—	—	—	—
21	15.8	15.5	14.4	15.4	0	9.3
22	14.7	18.4	15.4	18.3	0.7	9.6
23	16.0	16.3	18.5	13.9	0.1	11.6
24	15.6	12.3	14.8	14.8	0	9.6
25	15.1	—	15.8	17.8	—	9.4
Mean	15.14	15.05	15.64	14.88	0.75	9.78
Standard Dev.	2.55	2.72	2.17	2.17	0.67	1.60
Coefficient of Variation, %	16.9	18.0	13.9	14.6		16.4
Calculated Value	18	18	18	18	0	12
Mean as % of Cal. Value	84	83	87	83		82
26*	14.6	14.3	11.6	7.8	0.6	8.5

* Received too late for inclusion in summary.

RESULTS OF ANALYSIS

Tables 2 and 3 present the average values reported by twenty-five collaborators for the six samples using the two suggested methods of reading. The data of one collaborator was received too late for inclusion

TABLE 3.—*Average values found for vitamin A in collaborative samples*
Vitamin A values calculated from internal standard

COLLABORATOR	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6
1	17.3	18.9	17.4	16.4	1.1	11.8
2	18.7	17.2	18.8	18.6	1.4	12.7
3	11.2	10.1	11.1	8.5	0.3	6.5
4	16.7	13.5	15.7	14.9	0.5	10.2
5	22.2	21.5	23.5	12.8	1.7	10.1
6	14.3	14.5	18.7	13.9	0.7	9.1
7	18.7	18.7	18.3	18.6	1.1	12.4
8	15.5	18.3	19.1	15.8	—	—
9	17.0	16.8	16.2	17.0	0.3	10.7
10	—	—	—	—	—	—
11	19.2	14.8	19.4	17.3	0.9	11.1
12	22.8	27.2	30.6	30.6	5.1	16.6
13	20.0	21.3	20.0	19.1	0	11.3
14	12.7	12.0	25.8	18.0	6.1	8.1
15	18.9	15.5	15.5	15.3	1.0	9.7
16	15.0	17.8	18.4	19.1	1.1	13.6
17	20.8	14.5	21.1	16.9	0.7	9.5
18	14.1	11.6	18.0	12.5	0.8	12.3
19	15.9	12.5	18.3	14.3	0.8	7.5
20	23.1	20.9	26.2	25.3	4.8	12.5
21	20.9	19.2	15.0	16.6	0.7	11.0
22	14.0	20.3	14.8	23.6	0.8	9.1
23	—	—	—	—	—	—
24	16.6	12.6	15.5	17.2	0	9.8
25	15.4	16.9	18.2	17.8	1.3	9.7
Mean	17.43	16.81	18.94	17.40	1.42	10.70
Standard Dev.	3.27	4.00	4.28	4.50	1.66	2.15
Coefficient of Variation, %	18.8	23.7	22.5	25.9		20.1
Calculated Value	18	18	18	18	0	12
Mean as % of Cal. Value	97	93	105	97		89
26*	29.4	29.4	26.4	21.9	15.4	18.3

* Received too late for inclusion in summary.

in the statistical evaluation. Most of the values in Tables 2 and 3 represent the average of three individual analyses.

Table 4 presents the data submitted by alternative procedures.

SUMMARY OF COMMENTS OF COLLABORATORS

Collaborators were asked by questionnaire for their reaction to various steps in the method and a detailed questionnaire was utilized as a means of checking on possible variation in procedural details which were not fixed by the method submitted.

Of the twenty-six collaborators submitting results, twenty-four found the extraction procedure satisfactory. Several suggested some modification.

The chromatographic procedure was found to be satisfactory by sixteen col-

TABLE 4.—*Vitamin A in collaborative samples by alternative methods*

COLLABORATOR	NO. 1	NO. 2	NO. 3	NO. 4	NO. 5	NO. 6	METHOD
1	17.5	17.3	17.7	17.8	0	11.3	Method of Cooley, *et al.* (5)
12	25.2	21.2	21.2	21.1	2.1	14.2	Skelly B extr., $SbCl_3$ Reading
12	18.9	17.0	17.2	16.3	1.2	11.7	Skelly B extr., GDH Reading
12	20.0	17.6	18.2	20.3	1.1	14.1	30% Acetone extr., GDH Reading
23		19.0		16.2			10 ml instead of 25 ml volume before chromatography
25	11.7	17.3	17.5				Saponification—small tube chromatography
Mean	18.6	18.2	18.4	18.3	1.1	12.8	

laborators. Of the remainder several suggested changes and some did not reply to this question.

A total of twenty out of twenty-six collaborators found the colorimetric method of reading samples given in the procedure to be satisfactory. Five did not respond to this question. Several suggest modifications of the given procedure.

Of those responding, fourteen prefer the use of a standard curve, whereas eight prefer the use of an internal standard. The others expressed no preference.

It is of interest to note that seven different makes of instruments were represented among those submitting results. A breakdown of results by types of instrument used in reading showed no definite trends.

A compilation of the answers to questions on details of technique or equipment reveals the following:

Practically all of the collaborators carried out their extractions in all glass equipment with standard taper joints. Many checked the volume after extraction and of those, six out of thirteen found it desirable to make a volume correction. This is a possible source of error for those who did not check and is a weakness in the procedure as given.

In general artificial light of a subdued nature was used by the collaborators but only seven out of twenty-six analysts resorted to non actinic glassware. The results of the seven taking this precaution were not improved as a group.

The temperature of evaporation varied from room temperature to 100°C. A great majority but not all of those using temperatures in excess of 60°C. for evaporation of samples reported results below the average. This is a point which should be experimentally verified and is a possible source of low results. The use of vacuum during evaporation was nearly unanimous.

Eight analysts used an inert gas during or after evaporation with no noticeable improvement as a group.

Column inside diameters varied from 15 to 23 mm with most analysts using a packed depth of about 100 mm. Both cotton and glass wool plugs were used in about equal proportions at the bottom of the columns with equal results.

Sintered glass columns made with standard taper joints may give trouble by retaining a small portion of uneluted vitamin A in the absorbing material just about the joint and next to the outer wall. This space is not above a sintered glass area and is not easily eluted.

All collaborators used the prescribed adsorbent. A few reported moisture values which varied from 0.2% to 8.5%. The few values reported indicate that some attempt

may be necessary to control moisture since it is known to affect the adsorptive characteristics of many column materials. The highest moisture value reported was associated with erratic results and may have been a contributing cause. This point needs experimental verification.

A number of collaborators varied the exact composition of the antimony trichloride reagent with no apparent affect on the results.

INTERPRETATION OF RESULTS

In interpreting the results of this collaborative work it must always be kept in mind that vitamin A is an unstable material which can be readily destroyed in storage. For that reason the comparison of analytical values is constantly open to the hazard of vitamin A losses and false interpretations based on uncontrolled losses may be easily made.

By the use of suitable precautions in storage of the samples, and by the use of large numbers and statistical analysis of the data, these hazards may be brought to a minimum.

The results obtained by the different collaborators were first studied with respect to the date of analysis. No trend was evident so that it was assumed in future calculations that storage losses, if any, were at least random with respect to the different laboratories reporting.

In order to determine which factors would be worthy of more concentrated study, an analysis of variance was run on the individual assay data of collaborators 1, 2, 3, 4, 5, 6, 7, 9, 11, 16, and 17, which were the first collaborators to submit detailed assay data for triplicate assays of all samples by both methods. This statistical study was run on samples No. 1 to No. 4. inclusive which have the same calculated values for vitamin A.

TABLE 5.—*Analysis of variance*

SOURCE OF VARIATION	DEGREES OF FREEDOM	SUM OF SQUARES	MEAN SQUARE	SIGNIFICANCE
Analysts	10	1,349.8	134.98	Sig. at 1%
Samples	3	98.3	32.77	Sig. at 1%
Methods	1	171.7	171.70	Sig. at 1%
A-S	30	452.0	15.07	Sig. at 1%
A-M	10	85.7	8.57	Not sig. at 5%
S-M	3	30.2	10.7	Not sig. at 5%
A-M-S	30	72.0	2.40	
Duplicate Assays	170*	833.0	4.90†	

* Corrected from total of 176 by subtraction of 6 =no. of assays for which a value was supplied (original data missing).

† Used as measure of error.

The data summarized in Table 5 indicate that there are four highly significant sources of variation. These are the analysts, the method of reading, the difference in samples, and the interaction between analysts and samples. This is based on the use of the mean square of the duplicate samples as the measure of error. An examination of the analytical data

would readily reveal some of these sources of variation, but not all.

An examination of the data in Tables 2 and 3 reveals that all but one of the mean values found fall below the calculated values for the given sample. This is as it should be because there are undoubtedly some vitamin A losses in mixing and holding the samples for assay.

The mean values found by reading with an internal standard are higher in every case than those found by reading from a standard curve for corresponding samples. The mean values read from an internal standard also agree better with calculated values. This would at first appear to be an advantage until an examination of the data indicates that much of the higher mean value is caused by the presence of a few unusually high individual results which by their value must be assumed to be in error unless one is to concede non-uniform mixing of the sample. This can be shown to be highly improbable by a comparison of the standard deviations for the two methods of reading.

A comparison of standard deviations reveals a much greater scatter of values by the method using an internal standard as compared to values read from a standard curve. A close examination of the instrument readings on the different samples (not included in this report) revealed many cases in which a loss of the added vitamin A seemed evident. This resulted in an abnormally low increment of blue color for the added vitamin A which in turn produced abnormally high vitamin A results by calculation. The alternative explanation, that an inhibition of blue color takes place, is not substantiated by the results which are in many cases far above calculated values.

It is of interest to note that the coefficient of variations was essentially the same for the blind sample and for the four samples containing vitamin A at known calculated levels for each method of reading. The recovery of calculated vitamin A given by the expression, "mean as per cent of calculated value," in Tables 2 and 3 was essentially the same for both blind and known samples. These facts indicate no prejudice by the analysts.

In order to determine how the composition of the samples would influence the results, the results were paired by analysts and methods and tested for significant differences. By such a scheme approximately forty-five pairs of results were available for each comparison differing only in the composition of the sample. The analysis of variance had previously shown that significant differences should be evident.

Table 6 shows the variations between samples involved and the results of the statistical comparisons.

A comparison of results obtained on samples No. 3 and No. 4 indicates a highly significant difference. Lower values for vitamin A are obtained in the presence of alfalfa in ration No. 4. This may seem at first to be contrary to expectation. If the experience reported by the analysts is taken into account it becomes evident that the difference is probably

caused by the fact that a clean cut of the vitamin A fraction in the chromatographic column may be made when carotene is present as an indicator. This division is not as sharp when carotene is not present and the result is that more impurities are washed through the column. These in turn increase the amount of color obtained in the antimony trichloride reaction.

Some information as to the source of these impurities can be found by contrasting the degree of difference found between the pair No. 1 and

TABLE 6

SAMPLE NO.	1	2	3	4
Ingredients in common	65%	65%	65%	65%
Alfalfa	—	15%	—	15%
Wheat	15%	—	15%	—
Soybean Meal	20%	20%	—	—
Fish and Meat Meal	—	—	20%	20%
Calculated Vitamin A added (U.S.P. Units/g)	18	18	18	18
Mean (all values)	16.28	15.93	17.29	16.14

COMPARISON	DIFFERENCE IN MEAN	*t* VALUE*	CHANCE OF TWO SAMPLES BEING THE SAME
No. 1 vs No. 2	0.35	1.13	27%
No. 3 vs No. 4	1.15	2.74	1%
No. 1 vs No. 3	1.01	2.19	4%
No. 2 vs No. 4	0.21	.25	50%

* Degrees of freedom =44 or 45.

No. 2 versus the pair No. 3 and No. 4. Samples No. 1 and No. 2 differ by the presence of 15% alfalfa in place of 15% wheat with the balance of the ration essentially vegetable in origin. In this case, the vitamin A found in the ration is lower in the presence of alfalfa but to a lesser degree than in the case of rations No. 3 and No. 4. The difference between the two pairs is in the amount of animal products present. This observation is in line with the known fact that animal protein sources, such as fish meal and meat scrap, often yield materials which will give substantial antimony trichloride colored reaction products of non-vitamin A origin. Hence, when these materials are present and the chromatography is not clean cut, one would expect higher color values to result.

As further substantiation of this observation a comparison of samples No. 1 and No. 3, in which 20% soybean meal has been replaced by 20% animal products with no other change involved, reveals a significant increase in found vitamin A. In this case with no alfalfa, chromatography is not clean cut and the additional impurities derived from the animal products have maximal effect.

In contrast the same alteration of formula in the presence of 15% alfalfa in each sample (No. 2 vs. No. 4) where better chromatographic conditions exist, gives only a slight and therefore not significant increase in vitamin A value in the presence of animal products.

It is true that the above data is open to other interpretation, but most such alternatlves do not agree with known facts. For example, the differences observed might be explained as resulting from differences in vitamin A stability. Yet vitamin A is normally less stable in the presence of animal products than in a purely vegetable ration.

The above observations would appear to justify the thought that the presence of carotene, from alfalfa or otherwise, as an indicator pigment may improve chromatography. This in turn suggests the possibility of using an added indicator dye or pigment as a regular part of the chromatographic procedure. By a suitable choice it might be possible to secure one which would give visual indication of the complete elution of vitamin A and at the same time have light absorption properties such that a trace of the dye would not interfere with the vitamin A reaction reading.

Several collaborators submitted results by alternative procedures, Table 5. The data is too limited to evaluate thoroughly but one may note that the average values obtained agree well with calculated values and that the spread of the results in this table is reasonably narrow except for sample No. 1.

SUMMARY

A chemical method for the determination of vitamin A was subjected to collaborative assay in 1949, and results show a fair degree of agreement between laboratories. Several possible leads for the improvement of the method were indicated by this work. The findings in this first collaborative work on this problem indicate the need for further study of the method.

ACKNOWLEDGMENT

The fine cooperation of the collaborators and the assistance of Wm. Brew in planning and conducting this collaborative assay on the measurement of vitamin A in mixed feeds is gratefully acknowledged.

RECOMMENDATION*

The Referee recommends that the collaborative procedure be studied further with different samples and with some alteration of the method.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **33**, 43 (1950).

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REPORT ON VITAMIN B_1 (THIAMINE)

By O. L. KLINE (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

Three methods for the determination of thiamine appear in the Sixth Edition of *Method of Analysis*. Each has certain specific advantages and the recommendations with regard to each made to this Association last year were adopted (1). At this time further action is being recommended.

RECOMMENDATIONS*

GROWTH METHOD (2)

Last year this method was adopted as official, first action. Although the method is not in wide use it is important that it be retained as a method of reference for use when biological specificity of other recommended methods is in question. During the past year no collaborative work was conducted, but no criticisms of the procedure or objections to its final adoption have come to the Associate Referee.

It is recommended that the growth method for the determination of thiamine, as it appears in Sixth Edition, *Methods of Analysis* (3) be adopted as official.

FERMENTATION METHOD (4)

This microbiological procedure has the advantage of biological specificity and easy application, particularly to complex materials, without use of enzyme treatment. It is also of value in determining the amount of thiamine destruction that has occurred as a result of heating or improper storage. The method makes use of the stimulating effect of thiamine upon the rate of fermentation of fresh yeast cells. During the past year some difficulty has been experienced in obtaining a fresh yeast of ideal thiamine content for the test. Since the amount of fresh yeast used for this purpose is small it is not feasible to develop a special commercial production of it. In view of the variation in difference in gas produced in the control bottles containing 1 and 2 micrograms of thiamine with commercially available fresh yeast, it is necessary to change (in Sec. **36.41, b**) the required difference from 28 ml. to 20 ml. (5). This change does not affect the validity of the results obtained, and has little effect

* For report of Subcommittee A and action of the Association, see *This Journal*, **33**, 44 (1950).

upon the precision of the method when reasonable care is taken in reading the gas volumes.

It is recommended that with this modification, the fermentation method for the determination of thiamine be adopted as official, Details of the method will appear in the 7th Edition (1950) of *Methods of Analysis.*

THE FLUOROMETRIC (THIOCHROME) METHOD (6)

Last year it was recommended to the Association that the fluorometric procedure for thiamine be modified by changing the description of preparation of the sample solution (1). This involved no important change in handling the sample, but rather spelled out in greater detail the steps involved when the method is applied to a variety of biological materials. No adverse comment on this change has been heard during the past year Further, the procedure as modified has been applied in several hundred determinations in the Nutrition Laboratory of the Food and Drug Administration. This experience appears to be adequate basis for adoption of the modified fluorometric procedure as official.

It is recommended that the fluorometric method for the determination of thiamine, as modified, be adopted as official.

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REPORT ON RIBOFLAVIN (VITAMIN B_2) (1)

MICROBIOLOGICAL METHOD

By HENRY W. LOY, JR. (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

The microbiological method for the assay of riboflavin as presented at the meeting of the Association last year was adopted as first action (2).

Since the adoption of this method, it has been the experience of the Associate Referee that the range in riboflavin content of the tubes containing the sample solution may be extended to include 0.025 microgram of riboflavin per tube without affecting the accuracy of the method. This wider range of acceptable values is of advantage in the assay of materials of unknown potency in that it permits a wider choice for the selection of the required six tubes. A survey was made of the desirability of such a change, and written response in the affirmative was obtained from a number of analysts who had taken part in the earlier collaborative studies

of this method. No further work was undertaken this year, but the results of the last two years' collaborative studies (3) and the continued successful use of this method by many laboratories justifies the following consideration. With the above-mentioned modification of the acceptable range of riboflavin concentrations per tube, and with certain minor editorial changes (4), the method is suitable for adoption as official.

RECOMMENDATION*

It is recommended that the microbiological method for the assay of riboflavin (2), with editorial changes, and with the following modification, be adopted as official.

On page 107, under Determination, paragraph 5, on line 6 and on line 11, change "0.05" to read "0.025."

REFERENCES

(1) *This Journal*, **23**, 346 (1940); **24**, 413 (1941); **25**, 459 (1942); **26**, 81 (1943); **27**, 540 (1944); **28**, 560 (1945); **29**, 25 (1946); **30**, 79 (1947); **31**, 701 (1948); **32**, 105, 461 (1949).
(2) *Ibid.*, **32**, 105 (1949).
(3) *Ibid.*, **31**, 701 (1948); **32**, 461 (1949).
(4) These will be incorporated in *Methods of Analysis, A.O.A.C.*, Ed. 7, (1950).

REPORT OF RIBOFLAVIN (VITAMIN B_2) (1)

FLUOROMETRIC METHOD

By HENRY W. LOY, JR. (Food and Drug Administration, Federal Security Agency, Washington, D.C.), *Associate Referee*

The fluorometric method for the assay of riboflavin as presented at the meeting of the Association last year was adopted as first action (2). No further work was undertaken this year, but the results of last year's collaborative study (3) and the continued successful use of this method by many laboratories justifies the following consideration. With certain minor editorial changes (4), the method is suitable for adoption as official.

RECOMMENDATION*

It is recommended that the fluorometric method for the assay of riboflavin (2), with editorial changes, be adopted as official.

REFERENCES

(1) *This Journal*, **30**, 392 (1947); **31**, 701 (1948); **32**, 108, 461 (1949).
(2) *Ibid.*, **32**, 108 (1949).
(3) *Ibid.*, **32**, 461 (1949).
(4) These will be incorporated in *Methods of Analysis, A.O.A.C.*, Ed. 7, (1950).

* For report of Subcommittee A and action of the Association, see *This Journal*, 33, 43 (1950).

REPORT ON FOLIC ACID

By LAURA M. FLYNN (Dept. of Agricultural Chemistry, University of Missouri, Columbia, Mo.), *Associate Referee*

The microbiological method for determining folic acid was subjected to collaborative assay under the auspices of the Association of Official Agricultural Chemists, for a second time, in 1948. Nineteen groups, in commercial plants or in laboratories supported by Federal or State funds, gave generous aid in the 1948 study. Results from the various laboratories showed, in general very good agreement (1). It seemed wise, however, to subject the assay to further study before recommending a procedure for adoption as an A.O.A.C. "first action" method of assay of the vitamin. At the conclusion of the study last year two problems required clarification: (A) whether *L. casei* or *S. faecalis* should be selected as the test organism for the A.O.A.C. method, and (B) what steps should be taken in extraction procedures to bring results from microbiological determinations into harmony with results from chick tests. A few studies of extraction methods have been made in several laboratories. It did not appear, however, that data from these investigations were sufficiently complete to merit asking collaborators to test new extraction procedures at the present time. The directions sent to the collaborating laboratories were changed very little, therefore, from those used last year.

The materials chosen for assay in 1949 were (a) a solution of crystalline folic acid which would require no extraction before testing, (b) dehydrated turnip greens, (c) a desiccated liver concentrate, (d) soy flour (not the same material assayed last year), and (e) dehydrated mustard greens. (The mustard greens were the same material tested last year, but had been stored, sealed, at 10°F. during the last twelve months). The Associate Referee sent the samples with the request that the recipients assay any or all of them, as time permitted. Collaborators were asked to use the specified methods for the assay, but were told that the Associate Referee would be very grateful for folic acid values on the samples determined by any other methods they were accustomed to use, if time permitted them to do the additional work.

To insure as much uniformity as possible among the laboratories, the materials sent to the participants included (A) crystalline folic acid for the standard solution (Folvite 7–8969), (B) agar stab cultures of *L. casei* and *S. faecalis*, and (C) a small amount of desiccated chicken pancreas for the enzyme hydrolysis of the samples. Each collaborator was asked to use either or both organisms for his assays. The two organisms were sent out in the hope that the data and comments from collaborators this year would indicate clearly which organism is preferred by the chemists making these assays in the laboratories throughout the country. In the experience of the Associate Referee excellent assays can be made with

either organism. This was indicated, also, in the results of the 1948 study, and a recent paper by Jones and Morris (4) presents findings in harmony with this viewpoint. It seemed desirable, however, to make a selection this year of *one* organsim to be recommended for use in a "first action" A.O.A.C. method.

At the present time no material is available which is recognized as a reference standard for folic acid. In the absence of an official standard the synthetic pteroylglutamic acid sent to the collaborators (Folvite 7–8969) was checked by E. E. Pickett, of the Spectroscopic Laboratory of the Department of Agricultural Chemistry at the University of Missouri. A Beckman spectrophotometer was used in the measurement of extinctions at wave lengths 256, 283, and 365 millimicrons. $E^{1\%}_{1cm.}$ values were, respectively, 578, 559, and 194. The vitamin was tested at *p*H 11.1, at a concentration of 0.001%. These data characterize the material used as a standard by the collaborators. Physical constants for pteroylglutamic acid, including extinction data, have been published by Parke, Davis and Company (2) and by the Lederle Laboratories Division of the American Cyanamide Company (3). According to extinction coefficients published by Parke, Davis and Company, the material used by collaborators would be calculated to be about 93% pure. According to extinction coefficients established at the Lederle Laboratories, the material is 100% pure.

The medium specified for the 1949 collaborative study was that used in the 1948 study. It has been used successfully over a period of months in the laboratory of the Associate Referee. It differs from several other media frequently used in folic acid assays as follows: cysteine is substituted for cystine, peptone and alanine are omitted, there are minor changes in the vitamin mixture, the amount of $MnSO_4$ is increased, and "Tween" and glutathione are added. The medium is easily prepared from materials commercially available, is reproducible, and by altering the buffer it can be used successfully with either *L. casei* or *S. faecalis*. Responses of the bacteria in this medium can be evaluated either acidimetrically or turbidimetrically, whichever organism is used.

COLLABORATORS

The chemists co-operating in this study are listed below. Grateful acknowledgement is made of the generous and gracious help of all who took part in the endeavor.

O. D. Bird, Research Laboratories, Parke, Davis and Company, Detroit, Mich.
Colin R. Cameron, Dept. of Animal Nutrition, Ontario Agricultural College, Guelph, Canada.
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RESULTS

Assayists will appreciate the amount of work involved in the large number of microbiological tests represented in the data compiled here. Many laboratories submitted detailed protocols with their results, and the general excellence of the work recorded was obvious.

Results submitted by the co-operating laboratories are summarized in Tables 1–7. To facilitate comparisons, arithmetic means and standard deviations are listed in the tables. In the calculation of these means only one figure was used from each laboratory, an average of the results of the individual tests from each laboratory. Data from several laboratories were omitted from the calculations of the averages. Values from Laboratory 9 on turnip greens and soy flour were not included because they differed markedly from those from other laboratories. The data from Laboratories 17 and 18 were received after the computations were made.

The tables require few comments. Table 1 is a summary table and is made up of the means of the assay results, and their standard deviations. Results from microbiological assays of folic acid are the same, regardless of the organism chosen. When natural foodstuffs were assayed the standard deviations were larger than when the unknown solution of folic acid was assayed.

TABLE 1.—*Summary of mean values from microbiological assays in 1949 A.O.A.C. collaborative study of folic acid*

ASSAY METHOD	TEST ORGANISM	FOLIC ACID SOLN	MUSTARD GREENS	TURNIP GREENS	SOY FLOUR	LIVER CONCENTRATE
		mmg/ml	*mmg/gm*	*mmg/gm*	*mmg/gm*	*mmg/gm*
Microbiological						
Specified Procedure	*L. casei*	0.251 ±0.020	9.44 ±1.35	6.206 ±1.06	4.146 ±0.93	20.16 ±3.19
All assays	*L. casei*	0.252 ±0.017	9.59 ±1.36	6.265 ±1.12	4.26 ±0.85	20.30 ±2.89
Specified Procedure	*S. faecalis*	0.241 ±0.019	9.27 ±0.98	5.93 ±0.88	3.933 ±0.74	19.47 ±2.96
All assays	*S. faecalis*	0.2404 ±0.023	9.37 ±1.06	5.80 ±1.01	3.955 ±0.78	20.31 ±2.28
Biological (Chick assay)*						
Growth		—	—	14.3	3.2	—
Blood		—	—	13.6	4.9†	—
Average		—	—	13.95	4.0	—

* Contributed by Boyd O'Dell and Herbert Graff, Dept. of Agricultural Chemistry, University of Missouri.

† Value from blood test was considered better for soy flour.

The development of adequate extraction procedures is the biggest problem in microbiological assays of folic acid. According to chick tests made last year the desiccated mustard greens contain 13–14 mmg. of folic acid per gram. In contrast, the microbiological assays indicate that the mustard greens contain only about 9.5 mmg. per gram. This is disappointing, but one is more disturbed by the marked divergence between results of a chick test of desiccated turnip greens (14 mmg. of folic acid per gram) and the findings of the collaborative microbiological assays of the same material (about 6 mmg. per gram). The estimate from the hematocrit in a chick assay of the potency of the soy flour (4.9 mmg. per gram) was considered a better value than that calculated from growth (3.2 mmg. per gram). The mean value from the collaborative microbiological assays of the flour was about 4.1 mmg. per gram.

All data obtained by the prescribed method are shown in Tables 2 (*L. casei*) and 3 (*S. faecalis*). As a rule the agreement is good, although in some instances the variability is larger than might be expected.

The results in Table 4 were obtained with media or methods other than the one prescribed. It is quite evident that the folic acid potencies indicated are more variable than they were when the prescribed procedure was followed, but unfortunately one can not decide from the data which procedure is most reliable.

TABLE 2.—*Results from 1949 A.O.A.C. assays* (*Lactobacillus casei* in specified medium)

LAB. NO.	METHOD OF EVALUATION	FOLIC ACID SOLN	MUSTARD GREENS	TURNIP GREENS	SOY FLOUR	LIVER CONCENTRATE
		mmg/ml	*mmg/gm*	*mmg/gm*	*mmg/gm*	*mmg/gm*
1	Acidimetric	—	9.21	6.80	5.27	21.68
				6.34	4.24	
	Ave.	—	9.21	6.57	4.75	21.68
2	Turbidimetric	0.26	9.2	6.8	6.4	24.8
		0.265	10.4	6.8	6.2	27.0
	Ave.	0.263	9.8	6.8	6.3	25.9
3	Acidimetric	—	7.61	5.34	4.81	17.4
			8.19	5.2	4.16	16.7
	Ave.	—	7.90	5.27	4.49	17.05
4	Acidimetric	0.209	6.85	4.25	3.37	14.45
6	Acidimetric	0.25	8.80	6.67	4.00	18.17
9	Acidimetric	—	10.2	12.0*	9.4*	—
10	Turbidimetric	0.25	9.7	5.5	3.0	19.45
		0.28	10.2	5.0	4.2	20.0
	Ave.	0.265	9.95	5.25	3.6	19.73
12	Acidimetric	0.24	8.4	5.8	3.3	16.7
13	Acidimetric	0.257	11.1	7.6	3.6	22.6
14	Acidimetric	0.26	—	5.60	3.43	20.25
				5.32	3.24	
				6.50	3.47	
				6.46		
	Ave.			5.97	3.38	
15	Acidimetric	0.266	11.74	7.88	4.67	22.17
16	Turbidimetric	—	8.5	—	—	
			9.0	—	—	23.1
			9.9			
	Ave.		9.13			
	Mean	0.251 ±0.020	9.44 ±1.35	6.206 ±1.06	4.146 ±0.93	20.16 ±3.19
17	Acidimetric	0.337*	9.12*	5.79*	4.29*	22.7*
18	Acidimetric	0.254*	7.82*	4.98*	3.41*	15.8*

* Not included in averages (late).

TABLE 3.—*Results from 1949 A.O.A.C. collaborative study of folic acid assays (Streptococcus faecalis* in specified medium)

LAB. NO.	METHOD OF EVALUATION	FOLIC ACID SOLN	MUSTARD GREENS	TURNIP GREENS	SOY FLOUR	LIVER CONCENTRATE
		mmg/ml	*mmg/gm*	*mmg/gm*	*mmg/gm*	*mmg/gm*
1	Acidimetric	—	11.3	5.5	4.7	23.7
2	Results too poor to report					
5	Acidimetric	0.224	7.35	4.04	2.80	12.76
6	Acidimetric	0.25	8.74	6.45	4.90	19.72
7	Acidimetric	0.213	8.33	5.87	3.82	16.7
8	Turbidimetric	0.23	9.30	5.40	2.60	20.20
		—	8.30	5.10	2.60	21.40
	Ave.		8.80	5.25	2.60	21.40
9	Acidimetric	—	9.5	8.1	11.1*	—
10	Turbidimetric	0.25	9.3	5.46	4.09	19.66
		0.31	9.6	5.6	4.8	20.6
	Ave.	0.28	9.45	5.53	4.445	20.14
11	Acidimetric	0.227	—	5.857	4.468	18.93
12	Acidimetric	0.24	10.4	6.35	4.7	17.4
	Turbidimetric	0.25	—	—	3.82	—
	Ave.				4.26	
13	Acidimetric	0.255	9.4	5.9	3.7	22.5
14	(?)	—	—	5.60	3.46	22.45
15	Acidimetric	0.2486	9.37	6.66	4.74	16.94
16	Turbidimetric	—	8.83	6.25	3.15	23.2
			10.00	5.72	3.44	18.85
			10.00			
			8.6			
	Ave.		9.36	5.98	3.30	21.03
	Mean	0.241 ±0.019	9.27 ±0.98	5.93 ±0.88	3.933 ±0.74	19.47 ±2.96
17	Acidimetric	0.354*	10.1*	6.94*	4.05*	25.2*
18	Acidimetric	0.277*	9.51*	7.58*	6.05*	19.1*

* Not included in averages (late).

TABLE 4.—*Results from assays of samples by procedures other than those specified for 1949 A.O.A.C. study*

LAB. NO.	METHOD	METHOD OF EVALUATION	F.A. SOLN	MUSTARD GREENS	TURNIP GREENS	SOY FLOUR	LIVER CONCENTRATE
			mmg/ml	*mmg/gm*	*mmg/gm*	*mmg/gm*	*mmg/gm*
1	Specified procedure, own materials	*L. casei* Acidimetric	—	—	5.36	—	17.35
			—	—	6.52	—	—
	Ave.				5.94		
3	Specified procedure, own materials	*L. casei*	—	—	4.94	—	15.3
			—	—	4.2	—	16.7
	Ave.				4.57		16.00
12	Procedure of own lab.	*L. casei*	0.25	13.2	8.3	5.5	25.2
1	Specified procedure, own materials	*S. faecalis*	—	—	6.74		24.96
5	Difco dehydrated medium	*S. faecalis*	0.29	7.5	1.43	1.63	26.00
8	Teply & Elvehjem	*S. faecalis*	0.18	8.20	3.30	2.40	20.02
			0.20	—	6.20	4.56	—
	Ave.		0.19		4.75	3.48	
9	Difco dehydrated medium	*S. faecalis*	—	11.0	6.4	13.0*	—
12	Specified procedure, own enzyme treatment	*S. faecalis* Acidimetric	0.20	11.3	6.7	4.7	21.2
13	Difco dehydrated medium	*S. faecalis*	—	9.4	5.7	4.2	25.1
	Mean† Averages	*L. casei*	0.25(1)	13.2(1)	6.27	5.5(1)	19.52(3)
		S. faecalis	0.227(3)	9.48(5)	5.29(6)	3.5(4)	23.46(5)

* Not included in averages.
† No. of laboratories using the organism is shown in parentheses.

TABLE 5.—*Results from A.O.A.C. collaborative study of folic acid assays (average of determinations reported from each laboratory)*
(Test organism *Lactobacillus casei*)

LAB. NO.	NO. OF RESULTS AVERAGED	FOLIC ACID SOLN	MUSTARD GREENS	TURNIP GREENS	SOY FLOUR	LIVER CONCENTRATE
		mmg/ml	*mmg/gm*	*mmg/gm*	*mmg/gm*	*mmg/gm*
1	1–4	—	9.21	6.26	4.75	19.52
2	2	0.263	9.8	6.8	6.3	25.9
3	2–4	—	7.9	4.92	4.49	16.5
4	1	0.209	6.85	4.25	3.37	14.45
6	1	0.25	8.80	6.67	4.00	18.17
9	1	—	10.2	12.0*	9.4*	—
10	2	0.265	9.95	5.25	3.6	19.72
12	2	0.245	10.8	7.05	4.4	20.95
13	2	0.257	11.1	7.6	3.6	22.6
14	1–3	0.26	—	5.97	3.38	20.25
15	1	0.266	11.74	7.88	4.67	22.17
16	1–3	—	9.13	—	—	23.1
	Mean	0.252 ±0.017	9.59 ±1.36	6.625 ±1.12	4.26 ±0.85	20.30 ±2.89
17	1	0.245*	9.56*	5.93*	4.17*	23.85*
18	1	0.254*	7.82*	4.98*	3.41*	15.8*

* Not included in averages (late).

TABLE 6.—*Results from A.O.A.C. collaborative study of folic acid assays (average of all determinations reported from each laboratory)*
(Test organism *Streptococcus faecalis*)

LAB. NO.	NO. OF RESULTS AVERAGED	FOLIC ACID SOLN	MUSTARD GREENS	TURNIP GREENS	SOY FLOUR	LIVER CONCENTRATE
		mmg/ml	*mmg/gm*	*mmg/gm*	*mmg/gm*	*mmg/gm*
1	1–2	—	11.3	6.12	4.7	24.33
5	2	0.257	7.43	2.74	2.22	19.38
6	1	0.25	8.74	6.45	4.90	19.72
7	1	0.213	8.33	5.87	3.82	16.7
8	2	0.203	8.60	5.00	3.04	20.94
9	2	—	10.25	7.25	12.05*	—
10	2	0.28	9.45	5.53	4.45	20.14
11	1	0.227	—	5.857	4.468	18.93
12	3	0.23	10.85	6.53	4.41	19.3
13	2	0.255	9.4	5.8	3.95	23.8
14	1	—	—	5.60	3.46	22.45
15	1	0.249	9.37	6.66	4.74	16.94
16	1–4	—	9.36	5.98	3.30	21.03
	Mean	0.2404 ±0.023	9.37 ±1.06	5.80 ±1.01	3.955 ±0.78	20.31 ±2.28
17	1	0.354*	10.1*	6.94*	4.05*	25.2*
18	1	0.277*	9.51*	7.58*	6.05*	19.1*

* Not included in averages (late).

Each number in Table 5 is an average of the results of all the determinations reported by an individual laboratory, using *L. casei* as the test organism. The values shown in Table 6 are averages of the findings of all the determinations reported by each laboratory from assays with *S. faecalis* as the test organism. Only the figures from Laboratories 1, 3, and 12 in Table 5 include data from tests which were variations of the prescribed procedures and materials. The average values from these laboratories, as shown in Table 5, differed little from the averages from the same laboratories as presented in Table 2. In other words, all the folic

TABLE 7.—*Results of assays of one sample by same laboratory using same procedure in 1948 and 1949*
(Folic acid in mustard greens)

LAB. NO.	AS DETERMINED WITH *L. casei* IN A.O.A.C. MEDIUM		AS DETERMINED WITH *S. faecalis* IN A.O.A.C. MEDIUM	
	1948	1949	1948	1949
	mmg/gm	*mmg/gm*	*mmg/gm*	*mmg/gm*
1	8.76	9.21	—	11.3
2	9.3	9.8	7.3	—
5	6.57	—	5.13	7.35
6	8.0	8.80	10.50	8.74
8	—	—	4.8	8.80
9	9.3	10.2	—	9.5
10	10.0	9.95	12.4	9.45
13	7.32	11.1	7.38	9.4
15	—	—	7.88	9.37
16	9.67	9.13	10.45	9.36
Mean	8.62 ± 1.13	9.74 ± 0.72	8.23 ± 2.28	9.25 ± 0.97
17	8.1*	9.12*		
18	6.7*	7.82*	6.5*	9.51*

* Not included in averages (late).

acid assays done in any one laboratory, using *L. casei* as the test organism, gave comparable results. Among the data shown in Table 6 the results from Laboratories 1, 5, 8, 9, 12, and 13 include values found with procedures or materials other than those specified. Comparison of figures in Tables 3 and 6 reveals that as a rule the average folic acid potency of a sample, as determined in each laboratory in assays with *S. faecalis*, was the same, regardless of the method used. The data from Laboratory 5, however, did not show good agreement between results from assays by the prescribed procedure and assays by the other procedure used. Folic acid potencies of samples (mmg. per gram), determined by Laboratory 5, (a) by the specified procedure, and (b) by the other procedure used, were, respectively: Turnip greens, 4.04, 2.74; soy flour, 2.80, 2.22; liver concentrate, 12.76, 19.38.

Table 7 is a summary of the results of determinations of the same sample, mustard greens, by the prescribed procedure in 1948 and in 1949. The reproducibility of the results seems good, considering the limitations of microbiological methods for folic acid, and realizing the changes in personnel, supplies, and cultures that may occur in any laboratory in a year's time. It is satisfying to find that the standard deviations are less this year, and that there was less variation this year than last year between results of microbiological determinations and chick assays of the test material.

INFORMATION FROM QUESTIONNAIRES

An attempt has been made to summarize here pertinent information from the questionnaires returned in 1949. In some cases comments from the two earlier collaborative assays have been included. Each year the group participating in the study has included a large number of chemists experienced in microbiological assays of folic acid. Five of the eighteen chemists contributing to the 1949 investigation were, according to their own statements, inexperienced in the assays, but their comments and results proved to be valuable contributions.

Several laboratories experimented with different enzyme treatments. Laboratory 12 used its own method, the Fleischmann Laboratory modification of the method of Teply and Elvehjem (5) and the procedures of Burkholder, McVeigh and Wilson (6). (Takadiastase and chick pancreas were both used.) Table 4 includes the results obtained by this procedure. Laboratory 7 also contributed data from different routines of enzyme treatment. They subjected two samples to three enzyme treatments, (1) chicken pancreas preparation 24 hours at *p*H 7.2, (2) hog kidney preparation 24 hours at *p*H 4.5, and (3) chicken pancreas preparation 24 hours at *p*H 7.2, followed by hog kidney preparation 24 hours at *p*H 4.5. In each case 100 mg. of enzyme preparation was used per 1.0 gram of sample. Results of assays (in mmg. per gm.) after these treatments were, respectively: Liver Concentrate (A.O.A.C. 1949 Collab. Sample No. 5, (1) 16.7, (2) 11.5, and (3) 16.8; Dried Brewers Yeast, (1) 16.3, (2) 1.7, and (3) 16.8. They say, "These results are not in complete agreement with those reported by Sreenivasan, Harper, and Elvehjem (7) who used chick pancreas preparation followed by hog kidney enzyme prepared as described by Bird, *et al.* (8). We have not determined the enzyme activity of our hog kidney preparation which was a defatted preparation dried at 40°C. It is important, however, to make certain that bound forms available to the animal are completely hydrolyzed in the extraction procedure for microbiological assay. If enzyme preparations vary in their activity some means of standardization should be developed."

Purification of amino acids

It is essential that the casein hydrolysate used as a source of amino acid shall be free from folic acid. This is necessary in assays with either organism, but one cannot overemphasize the importance of rigid adsorption procedures in preparing a solution of casein hydrolysate for use in folic acid assays with *L. casei*. Several collaborators reported that they were able to get suitable blanks only after subjecting the commercial casein hydrolysates they were accustomed to use to adequate adsorption treatments. The Associate Referee has found it convenient to use Darco

60 at *p*H 3.0 to adsorb the folic acid. (Drs. Couch and Richardson recommend heating the Darco for two hours at 100° before use, and carrying out the adsorption by stirring at 80°C. for an hour.

William Popper, Jr., called our attention to another possible difficulty. He traced his trouble in a disappointing assay with *L. casei* to the l-tryptophane which (as it came from the supplier) was contaminated with folic acid. After treating the tryptophane solution with Norite (1 hour at *p*H 3.0) his blanks were extremely low (no acid produced without added folic acid).

L. casei vs S. faecalis

In the collaborative assays the medium used with *L. casei* was a variation of that used with *S. faecalis*. For use with *L. casei* the sodium citrate was omitted from the medium, the amount of K_2HPO_4 was increased to 0.5 gm. (for 100 tubes), and 0.5 gm. of KH_2PO_4 and 20 gm. of sodium acetate (anhydrous) were added. The number of bacteria in the inoculum in the assays with *L. casei* was reduced to one-tenth the number used in the assays with *S. faecalis*.

Of the commercial laboratories 8 prefer *L. casei* and 6 prefer *S. faecalis;* of the university laboratories 4 prefer *L. casei* and 3 prefer *S. faecalis;* of the two Federal laboratories participating in the study one preferred *L. casei* while the other preferred *S. faecalis*.

Unfortunately, few of the collaborators seem to have had extensive experience with *both* methods. The results of the 1948 and 1949 collaborative studies seem to indicate that *S. faecalis* is adequate for use in control work, and it offers the advantage of a short incubation period if turbidity is used as the criterion of bacterial response. It is possible that the use of *L. casei* offers important advantages in some assays, particularly in research work, if the experimental conditions are properly controlled. Several collaborators, however, reported that they were unable to perform satisfactory assays with *L. casei*. It is clear that it is more difficult to obtain consistently good results with *L. casei*. Because of the complications inherent in the use of the *L. casei* procedure, it would seem unwise to make the use of this organism mandatory.

SUMMARY

The microbiological method for determining folic acid was subjected to collaborative assay in 1949. Whether *L. casei* or *S. faecalis* was used in the medium suggested by the Associate Referee, and whether response of the test organism was measured turbidimetrically or acidimetrically, assay results on 5 samples were in excellent agreement. The use of either of the bacteria in other media gave results in harmony with those found in the medium suggested by the Associate Referee.

The folic acid value of the soy flour, determined microbiologically, was close to the value determined in the chick assay. Results of the micro-

biological assay and the chick assay of the turnip greens were not in good agreement. This indicates the need for further study of extraction procedures.

RECOMMENDATION*

The Associate Referee recommends for first action adoption of the microbiological assay of folic acid the method subjected to collaborative assays in 1948 and 1949, using *S. faecalis* as the test organism. Details of the method will be published in the 7th Ed., *Methods of Analysis* (1950).

ACKNOWLEDGMENTS

California Vegetable Concentrates, Inc., 2067 Clarendon Ave., Huntington Park, California, generously supplied the mustard green powder and the turnip leaf powder used in this study, while the soy flour was donated by the A. E. Staley Manufacturing Company of Decatur, Illinois.

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REPORT ON NICOTINIC ACID (NIACIN) OR NICOTINAMIDE (NIACIN AMIDE) (1)

MICROBIOLOGICAL METHOD

By O. L. KLINE (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

A revision of the official, first action method for the assay of nicotinic acid as presented at the meeting of the Association last year was approved (2). No further work was undertaken this year, but the continued successful use of this method by many laboratories justifies the following recommendation. With certain minor editorial changes (3), the method is suitable for adoption as official.

* For report of subcommittee A and action of the Association see *This Journal*, **33**, 44 (1950).

RECOMMENDATION*

It is recommended that the microbiological method for the assay of nicotinic acid (2), with editorial changes, be adopted as official.

REFERENCES

(1) *This Journal*, **27**, 105 (1944); **30**, 82 (1947); **32**, 110 (1949).
(2) *Ibid.*, **32**, 110 (1949).
(3) As will be incorporated in *Methods of Analysis*, A.O.A.C. Ed. 7 (1950).

REPORT ON VITAMIN D IN POULTRY FEED SUPPLEMENTS

By LEO FRIEDMAN (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

The biological assay for vitamin D in poultry feed supplements was first adopted as a tentative method by the A.O.A.C. in 1934 (1). Subsequent collaborative studies (2, 3, 4, 5, 6, 7, 8) have demonstrated the validity of the test, but have also emphasized the need for improvements that will allow greater precision and greater reproducibility between laboratories. Although the method as it now stands has not been subjected to collaborative study since 1941 (8), it is generally accepted and used as the most reliable method of assessing the potency of vitamin D in poultry feed supplements. It is recommended, therefore, that it be adopted, first action.

In 1942, Baird and McMillan (9) proposed the use of toe ash as a measure of response to vitamin D supplementation. Reports from other laboratories (10, 11) have corroborated Baird and McMillan's observations. Experience in the Referee's laboratory during the past year also indicates that the use of the toe ash may be a valid measure of response to vitamin D supplementation. The A.N.R.C. is conducting a study of the toe ash method, but at the time this report was prepared there was no information available. The toe ash procedure offers several possible advantages in simplifying the determination and it is felt that it should be carefully studied with a view to its adoption as an alternate method.

In 1936, Olsson (12) reported that the amount of vitamin D in chick rations could be related, within certain limits, to the width of the tarsal-metatarsal (tmt) distance measured in radiographs of the growing bones. He considered this a sensitive and satisfactory assay method. This technique has been widely applied in Europe and Canada and is claimed to be comparable in accuracy and precision of results with the A.O.A.C. bone ash method (13, 14). The technique has several desirable features, and during the discussion at the meeting last year it was suggested that laboratories carrying out vitamin D chick assays investigate the possi-

* For report of Subcommittee A and action of the Association, see *This Journal*, 33, 88 (1950).

bilities offered by the radiographic techniques. During the past year, the X-ray method was applied to the chicks on the A.O.A.C. assay in the Referee's laboratory. The results of many such comparisons indicate fairly good agreement for potencies calculated from bone ash and tmt values. It is suggested that collaborative work be carried out to determine the suitability of the X-ray method as an alternate procedure in measuring the response to vitamin D supplementation.

There has been much discussion at previous A.O.A.C. meetings of the desirability of incorporating into the method a procedure for statistical interpretation of the results of an assay, and criteria for determining the validity of an assay. An attempt to develop procedures for statistical evaluation will be made so that they may be included as part of any study to be conducted this coming year.

During the past year a new standard for vitamin D, namely, crystalline vitamin D_3, has been studied collaboratively on an international scale, as discussed by Dr. Nelson (15). This new international standard for vitamin D has been adopted by the World Health Organization and recommendation for adoption of a similar standard by the U.S.P. has been made by the Vitamin Advisory Board. Since the U.S.P. Reference Cod Liver Oil will not be available after the vitamin D_3 standard becomes official it becomes necessary to make provision for the use of the vitamin D_3 standard in the A.O.A.C. chick assay. Since four A.O.A.C. chick units have been found to be equivalent of three international units it will become necessary either to change the name of the A.O.A.C. chick unit or to reevaluate it. In order to avoid confusion with the earlier literature it has been suggested that a new name would be desirable and that the term "International chick unit of vitamin D" replace the A.O.A.C. chick unit.

RECOMMENDATIONS*

It is recommended—

(1) That the method for vitamin D in poultry feed supplements, as it appears in the *Methods of Analysis, A.O.A.C.* (16), be made first action.

(2) That recommendations 2a and 2b shall become effective at the time the U.S.P. Vitamin D_3, Reference Standard becomes official.

2a. That the term U.S.P. Reference Cod Liver Oil be deleted wherever it appears in the method and the term U.S.P. Vitamin D_3 Reference Standard replace it.

2b. That the term A.O.A.C. chick unit be deleted wherever it appears in the method and the term International chick unit replace it.

(3) That the toe ash and X-ray procedures be studied to determine their suitability as alternate procedures, and that an attempt be made to develop suitable procedures for interpreting the results and determining the validity of an assay.

* For report of Subcommittee A and action of The Association see *This Journal*, 33, 44 (1950).

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(13) *Analyst*, **65**, 326 (1940).
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(15) *This Journal*, **32**, 801 (1949).
(16) *Methods of Analysis, A.O.A.C.*, p. 625, 6th Ed. (1940).

REPORT ON CAROTENE

By F. W. QUACKENBUSH (Department of Agricultural Chemistry, Purdue University Agricultural Experiment Station, Lafayette, Indiana), *Associate Referee*

In response to reports from a number of sources that different laboratories were obtaining widely different analytical results on the carotene content of the same sample of alfalfa meal, a questionnaire was sent to 110 laboratories to determine whether a monthly check sample was needed. Replies to the questionnaire indicated that seventy-one laboratories wished to receive such a sample and that most of them were willing to pay the costs involved. Accordingly, arrangements were made to handle a collaborative check sample on a temporary basis at no charge, John Kephart of the National Dehydrating and Milling Company, Lamar, Colorado, having agreed to send out samples and the Associate Referee to handle tabulation and reporting of results. The first sample was sent out on June 15, 1949. Reports were received within two weeks from 38 collaborators on Sample 1 and from 62 collaborators on Sample 2.

Results tabulated on Sample 2 showed considerably less variation between laboratories than those on Sample 1. The mean value for Sample 2 was 85.0 mg. of carotene per pound; range, 64.0 to 102.6; average per cent deviation from the mean, 5.9. Using the same method on roughly comparable samples in 1948, the average per cent deviation was 6.0, and in 1947, 6.2.

The factors responsible for these deviations have not been ascertained. A study of data collected has not revealed any evident single cause.

Seven different instruments were used in measuring the carotene content of the final solution. Twenty-five laboratories reported values obtained with the Beckman DU spectrophotometer at a wave length of 436 millimicrons; six reported for the Coleman Universal spectrophotometer, settings from 435 to 440, four at higher wave lengths; eight, for the Evelyn colorimeter with 440 filter, one at 420; seven, for Klett Summerson, 440 filter; and eleven for other instruments. The average per cent deviation from the mean in reports from Beckman users was 5.5, from all others 6.1. Since this small difference is probably not significant, it is doubtful whether the instrument is an important factor.

Errors in calibrating instruments and converting readings to carotene values are undoubtedly responsible for some of the variations. In an attempt to check this, collaborators were asked to report readings with .02 per cent potassium dichromate solution. The mean carotene equivalent of these readings taken by the fifteen Beckman users who reported was 1.61, average per cent deviation 2.5. The mean carotene value reported for the alfalfa meal sample by the same fifteen collaborators was 86.9, average per cent deviation 4.4. Since the deviations on the dichromate and alfalfa meal values were not closely correlated, is it evident that other important variables are to be sought. Aqueous potassium dichromate is far from ideal as a standard solution for calibration. A standard of carotene stabilized in oil or of some other compound of similar spectral and solubility characteristics which would not be subject to spectral changes due to *p*H fluctuations would be much more suitable. Efforts should be continued to provide such a standard.

The use of overnight extraction at room temperature was compared with the one-hour hot extraction of the official method by six collaborators. Three obtained slightly higher results with the official method, and the other three, equal or higher with overnight extraction. The mean values were 86.5 for the official method and 87.5 for overnight extraction methods. Average deviations from the mean, 86.5, obtained by these analysts was 2.8. It is felt that these results warrant the recommendation that overnight extraction be specified as an alternate extraction procedure in the official method.

Variations in the adsorbent and contaminants in the solvent used in chromatography are probably the most important sources of error. While the magnesia specified in the method seems to be a fairly uniform product, a few collaborators have reported that it failed to hold noncarotene pigments in a clear-cut band above the carotene under the conditions specified. This may be due to variations in lots of the adsorbent when manufactured or to conditions under which it is stored in the laboratory. For example, it is well known that the adsorptive properties of such a material are altered by changes in moisture content. It would seem that an effort should be made to find another brand of magnesia which would have the same adsorptive properties and which could be

used alternatively as an adsorbent for carotene analysis. Also, a study should be made to define conditions under which magnesia can be stored in the laboratory without loss of adsorptive effectiveness.

Contaminants in the solvent also seem to have been responsible for some erroneous results, since some laboratories which reported difficulties seem to have obtained satisfactory results after they were advised to check the acetone for presence of alcohol or water. Dry, alcohol-free acetone should be specified in listing reagents. The hexane could also be a source of troublesome contaminants although this would seem less probable.

Finally, mention should be made of the variations in loss of carotene for the different samples during mailing and storage prior to analysis. The two-week maximum which is specified between dates of shipment and analysis seems to be ample for most laboratories, although some have reported arrival of the sample on the thirteenth day. However, samples held under the various conditions en route and in laboratories in different parts of the country can hardly be expected to deteriorate at the same rate. To minimize the temperature differences between samples, all collaborators were advised not to refrigerate samples after they were received. Some laboratories which received their samples promptly have reported substantial decreases of carotene in one week's time during the two-week period. Others have reported no substantial change. There seems to be no easy way to eliminate this variable. A request that all collaborators make analyses on the same day (*e.g.*, the fourteenth day after mailing) would probably not meet with general favor. To send all samples by air mail, especially if packed in dry ice, with instructions to refrigerate on arrival, would also be an effective but expensive improvement. It would seem that the analytical problems concerning adsorbent, solvent, and instrument standardization should receive attention first.

RECOMMENDATIONS*

It is recommended—

(1) That after modification (a) to include more rigid specifications on reagents, (b) to include overnight extraction at room temperature as an alternative to refluxing one hour, and (c) to provide for expression of results in mg. of carotene per pound, the method be adopted as official. The modified method is presented in detail below.

(2) That efforts be made to provide more suitable standard for calibration and checking of instruments.

(3) That efforts be made to provide means of continuing monthly check sample for at least a year.

(4) That the work on carotene analysis be continued along the following lines:

a. Selection of a suitable adsorbent as an alternative to that now specified.

* For report of Committee A and action of the Association, see *This Journal*, 33, 43 (1950).

b. Establish what conditions are suitable for storage of adsorbents in the laboratory.

c. Provide a method for purification of solvents to assure effective chromatographic separation.

CAROTENE IN HAYS AND DRIED PLANTS

REAGENTS

a. Acetone, dry alcohol-free.

b. Commercial hexane (b.p. 65–68°) (obtainable from Virginia Gas and Oil Company, Charleston, West Virginia. Skellysolve B (b.p. 60–70°) Skelly Oil Company, Kansas City, Missouri, is suitable if freshly distilled).

c. Activated magnesia (Micron brand No. 2642) (obtainable from Westvaco Chlorine Products Co., Newark, California).

d. Diatomaceous earth (*Hyflo Supercel*) (obtainable from Johns-Manville, Chicago, Illinois).

e. Sodium sulfate (anhydrous).

f. beta-carotene, chromatographically pure, crystalline reagent.

APPARATUS

a. Extractor, ca. 250 ml flask equipped with an efficient condenser (Goldfisch, Bailey-Walker, or ASTM extractor is suitable if no thimble is used).

b. Chromatographic tube 20 mm I.D.×175 mm long. A suitable tube can be made from a Pyrex test tube 22 mm O.D.×175 mm by sealing a smaller tube (ca 10 mm O.D.) in the bottom.

c. Spectrophotometer or other photometer suitable for measuring color density at or near wavelength of 436 millimicrons.

EXTRACTION

Hays and dried plants.—Grind the sample to pass a 40-mesh sieve. Weigh accurately a 2 g sample (1 g, if carotene content is high, four if low) and place in the flask of the extractor. Add 30 ml of a 3:7 mixture of acetone and commercial hexane (therefore, 30% acetone) to the flask and reflux the contents at a rate of 1–3 drops per second for one hour, or stopper and set in the dark at room temp. overnight (not less than 15 hours). Decant or filter the cold (room temp.) extract into 100-ml volumetric flask, wash the residue with hexane, and dilute the soln to volume. The soln, which now contains 9% acetone, is chromatographed.

CHROMATOGRAPHY

Prepare a chromatographic column with a 1:1 mixture of activated magnesia and diatomaceous earth. To prepare the chromatogram place a small plug of glass wool or cotton inside the tube, add the loose adsorbent to a depth of 15 cm, attach the tube to a suction flask, and apply the full vacuum of a water pump. Use a flat instrument such as an inverted cork mounted on a rod to gently press the adsorbent and flatten the surface. The packed column should be about 10 cm deep. Place 1 cm layer of anhydrous sodium sulfate above the adsorbent.

With the vacuum continuously applied to the flask pour the extract into the chromatographic column and use 50 ml of a 1:9 mixture of acetone and hexane to wash the carotene into the adsorbent and develop the chromatogram. Keep the top of the column covered with a layer of solvent during the entire operation. This is conveniently done by clamping an inverted volumetric flask full of solvent above the column with the neck 1–2 cm above the surface of the adsorbent.

The carotenes pass rapidly thru the column and the entire eluate is collected. Bands of xanthophylls, carotene oxidation products, and chlorophylls should be

present in the column when the operation is complete. Transfer the eluate, which has been reduced in volume by loss of vapor thru the water pump, to 100-ml volumetric flask and dilute to volume with a 1:9 mixture of acetone and hexane. The carotene content of this soln is determined photometrically.

PHOTOMETRY

Determine the density of the soln as soon as possible with a spectrophotometer at 436 millimicrons, or with some other instrument which is provided with a suitable filter system, such as the Klett photometer with a No. 44 filter or the Evelyn photoelectric colorimeter with a 440 filter. These instruments are first calibrated with solutions of *beta*-carotene of high purity as shown by the characteristic absorption curve. (See Beadle & Zscheile, *J. Biol. Chem.*, **144**, 21 (1942) for characteristic curve.) A calibration chart is prepared and the density of the soln to be determined is then converted into carotene concentration by referring to the chart.

When determinations are made with the Beckman spectrophotometer at 436 millimicrons, the specific absorption coefficient, 196, is used in the formula

$$C = \frac{\log \frac{I_0}{I} \times V \times 454}{196 \times L \times W}$$

where C is concentration of carotene in milligrams per pound contained in the original sample, V is the final volume of the eluate at time of reading, L is length of cell in cm, and W is the weight of sample.

Report results as milligrams of *beta*-carotene per pound.

A contributed paper, entitled "A New Standard for Vitamin D," by E. M. Nelson was published in the November, 1949, issue of *This Journal.*

A contributed paper, entitled "Protection of Riboflavin from Destructive Light Rays during Analysis" by R. T. Ottes and Floyd Roberts, was published in *This Journal,* November, 1949.

A contributed paper, entitled "A Study of Specificity of the Indophenol and the 2,4-dinitrophenylhydrazine Methods for Determining Vitamin C Values in Foods as Compared with the Crampton Bioassay Procedure," was presented at the Meeting. The complete report will be published in the Department of Agriculture Bulletin, of the Bureau of Human Nutrition and Home Economics.

Two papers entitled, respectively, "Stereoisomeric Analysis of Beta-Carotene," by E. M. Bickoff, M. E. Atkins, G. F. Bailey, and Fred Stitt, and "Determination of Beta-Carotene Stereoisomers in Alfalfa" by E. M. Bickoff and C. R. Thompson, were published in *This Journal,* November, 1949.

REPORT ON SOILS AND LIMING MATERIALS

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

The present report serves to convey the reports and recommendations from the Associate Referees and to record concordance therewith. With the exception of a study of procedures for the recovery of additive fluorine, which is presented in this number, page 653, the contribution from the Referee has been limited to suggestions to the Associate Referees.

RECOMMENDATIONS*

It is recommended—

(1) That the methods for sampling, **1.1–1.2,** and **3.1–3.2,** be made procedures, and that the following methods be made first action.

Methods for soils, **1.3–1.53.**
Neutralization value, **3.3–3.4.**
Carbon dioxide, **3.7.**
Total calcium oxide, **3.8.**
Total magnesium oxide, **3.9.**
Mechanical analysis of ground limestone, **3.10.**
Neutralization value of calcium silicate slags, **3.11.**

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

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

(4) That the utilization of carmin as an indicator in the determination of boron content of soils be studied further and that p-nitrobenzenazo-1, 8-dihydroxy naphthalene-3, 6-disulphonic acid, or "chromotrope-B" be studied as a suitable reagent for the determination of boron in soils.

(5) That further studies of *p*H in soils of arid and semi-arid regions be based upon soil systems of moisture content representative of an air-dry condition.

(6) That a study be made as to the adequacy of calcium hydroxide as a fixative for fluorine in soil charges of 1 to 1 proportion, with calcination at 500°C in 5- to 60-minute periods.

(7) That the direct distillation of unignited soil with sulfuric acid at 165°C, followed by distillation of an aliquot with perchloric acid at 135°C, be studied collaboratively.

(8) That the "2-point" barium hydroxide-barium acetate titration procedure for the determination of exchangeable hydrogen in soils be studied further in relation to calcite equilibria in a variety of soils.

(9) That the survey and comparison of methods for the determination

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* For report of Subcommittee A and action of the Association, see *This Journal,* 33, 42 (1950).

of phosphorus (a) that fraction in "available" state and (b) the proportions of organic-inorganic forms therein, be continued (*This Journal*, **30**, 43).

(10) That the survey and comparisons of methods for the determination of exchangeable potassium in soils be continued (*This Journal* **30**, 44).

(11) That the Associate Refereeship on exchangeable calcium and magnesium be maintained.

FLUORINE LOSSES IN THE CALCINATION OF ANALYTICAL CHARGES OF FLUORIDE-ENRICHED SOILS*

By W. H. MACINTIRE (*Referee*), L. S. JONES, and L. J. HARDIN (The University of Tennessee Agricultural Experiment Station, Knoxville, Tenn.)

Previous findings served to establish the fact that charges of experimentally phosphated soils should not be calcined beyond 500°C., as preparatory to the determination of their fluorine content by means of sulfuric acid distillation and the successive perchloric acid distillation from the concentrated sulfuric acid distillates (2). Moreover, those findings indicated that the inclusion of calcium caustics and calcination was not necessary in the determination of fluorine residual from substantial incorporations of rock phosphate and from repetitive annual incorporations of calcium fluoride.

The primary objective of the present experiments was to determine whether absolute recovery of fluorine could be assured through the double distillation procedure on lysimeter soils that had received incorporations of fluorides without companion inputs of phosphates. The considerations were (A) whether double distillation from the soil charge should be preceeded by calcination, (B) effects of variation in proportion of soil and lime in the charges before their preparatory calcination, (C) whether failures of double distillations to effect complete recoveries of additive fluorine from 500°C. and 900°C. calcines of limed charges of soil were due to thermally-induced fluoric complexes resistant to feasible distillation technique, or to the volatilization of fluorine from such charges during their calcination, (D) whether such calcinations induce volatilization of calcium fluoride, and (E) whether the magnesium content of dolomite is more potent than its calcium content in causing fixation of additive fluorine in the calcines of rationally dolomited soils or in accentuating volatilization of fluorine during the preparatory calcination.

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* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 10–12, 1949, as part of the report of the General Referee on Soils and Liming Material.

EXPERIMENTAL

Double distillation procedure.—To test for reproducibility of analytical results through "double distillation" from unheated and unlimed charges of soil, the samples used were 100-mesh reserves from a 4-year lysimeter experiment in which heavy incorporations of barium silicofluoride had been made initially, and also annually, alone and with limestone and with dolomite (3). Through parallel distillations it had been found that no increase in fluorine evolution was obtained in the distillations from the reserves that were ground to minus 325-mesh.

The analytical procedure was to distill from 1-gram charges by means of 50 ml. of sulfuric acid (1+1) at 165°C. in 125 ml. flasks, distillation being so regulated as to require an hour for the collection of 250 ml. in an excess of sodium hydroxide. The 250-ml. distillate was evaporated almost to dryness, and transferred to a distillation flask to obtain a 200-ml. 1-hour distillate from perchloric acid, plus silver sulfate, at 130°C., and aliquots thereof were titrated against 0.02 normal thorium nitrate, with .01 per cent solution of alizarin red S. as indicator (see addendum, p. 663).

TABLE 1.—*Reproducibility of fluorine recoveries through direct double distillation[a] from soils that had received inputs of barium silicofluoride*

SOIL NO.	BARIUM SILICOFLUORIDE INPUTS	FLUORINE FROM SOILS			
	lbs. per 2,000,000 of soil	*1*	*2*	*3*	*Ave.*
		p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*
612	None	250	238	250	246
613	Single inputs of 1500 lbs[b]	402	460	416	426
614	Four 1500-lb annual inputs[c]	1080	1060	1000	1047
615	Single 1500-lb input +limestone[b]	462	492	454	469
616	Single 1500-lb input+dolomite[b]	446	519	472	486

[a] H_2SO_4, 1 hour at 165°C., 250 ml from raw soil; $HClO_4$, 1 hour at 130°C.; 200 ml from the concentrated H_2SO_4 distillate.
[b] Equivalent to 304 p.p.m. of fluorine in soil.
[c] Applied annually, four consecutive years: Equivalent to 1216 p.p.m. in soil.

The triplicated findings given in Table 1 do not reflect fully satisfactory reproducibility in the results obtained by means of the double distillation procedure on the aged lysimeter soils that had received inputs of barium silicofluoride. Moreover, the several values do not tell how complete were the recoveries from the respective inputs of fluorine, since fluorine outgo in the rainwater leachings had been determined only for the fourth experimental year, shortly after the publication of the Willard-Winter method (4). Hence, the question as to completion in the recoveries of fluorine from inputs of fluorides will be considered further in the present text.

Effect of additive calcium oxide upon fluorine recovery from the 500°C. calcines of the limed charges of soil was tested through the distillations specified in Table 2, on the soils that were reported upon in Table 1.

The 1-gram charges of soil were limed at ratios of 1:0.7 and 1:3; the soil lime charges were wetted to slurries and dried in an electric oven and then calcined 30 minutes at 500°C. in an electric furnace. All succeeding calcinations were also made in the electric furnace.

The evolutions of fluorine from the soil (No. 612), as recorded in Table 2, do not register positive effect from the inmixed lime. The calcinations with the smaller inclusions of lime appeared to have induced slight, if any, increase in the recoveries from the soils that had received the incorporations of barium silicofluoride, whereas the larger proportion of

TABLE 2.—*Recoveries of fluorine through double distillation of analytical charges of soil plus CaO at two rates after 30-minute heatings at 500°C.*[a]

LYSIMETER SOIL NO.	CHARGES OF SOIL:CaO	FLUORINE CONTENT OF SOIL			
		1	*2*	*3*	*Ave.*
		p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*
612[b]	1:0.7	272	240	—	256
613		529	560	—	545
614[d]		1112	1060	—	1086
615[e]		460	492	—	476
616[f]		540	504	—	522
612[b]	1:3	296	291	—	294
613		333	450	360	381
614[d]		880	880	864	875
615[e]		400	384	400	395
616[f]		323	308	440	357

[a] Distillations from H_2SO_4 @ 165°C., and $HClO_4$ at 130°C. as in footnote (a) of Table 1 for the lysimeter soils that had received the specific inputs of $BaSiF_6$.
[b] No input of $BaSiF_6$.
[c] Single incorporation of 1500 lbs. of $BaSiF_6$ per 2,000,000 pounds of soil.
[d] Four 1500-lb annual incorporations of $BaSiF_6$.
[e] Single 1500-lb incorporation of $BaSiF_6$ as in (c): plus limestone, 2 tons.
[f] Single 1500-lb incorporation of $BaSiF_6$ as in (c): plus dolomite, 2-ton $CaCO_3$ equivalence.

inmixed lime induced significant diminution in the recoveries of fluorine from those soils.

Effect of duration of calcination. Analytical charges of the fluoride-fortified soils of Tables 1 and 2 were mixed with calcium oxide in equal proportion and subjected to 5-minute and 60-minute calcinations. From the intra comparisons of the data of Table 3, in relation to the results obtained for the 1-hour direct calcinations, as in Table 1, and for the 30-minute calcination of the limed charges, as in Table 2, it appears that 5 minutes is of sufficient duration for the heating of soils of the type used in the present experiment. Apparently also, the longer calcination tended to diminish the recoveries. This is upon the assumption that it is necessary that the charges be limed and incinerated prior to their being subjected to double distillation.

Effect of protracted sulfuric acid distillation, in relation to complete

recovery of additive fluorine, was registered through four successive 2-hour distillations of 250 ml from the control soil No. 612 and soil No. 614, which had a high content of additive fluorine. Since the perchloric acid distillations from the last three of those 250-ml distillates were found to be virtually devoid of fluorine, it is established that duration of 2 hours and distillates of 250 ml are adequate to assure maximal removals by means of the sulfuric acid distillation.

Comparisons of fluorine recoveries through the use of different over-all procedures, and by different analysts at different times, are given in Table 4 for the fluoride-fortified soils of Tables 1, 2, and 3. Incidence of residual fluorine ascribed to each fluoride-fortified soil was computed as the sum of the quantity registered by double distillation for the control soil in 1949, plus the respective amount of fluorine that was incorporated into the fluoride-fortified soil.

TABLE 3.—*Influence of duration of the heating of lime-fortified soil charges*[a] *upon fluorine recoveries by means of double distillation*[b]

SOIL NO.	5-MINUTE HEATING				60-MINUTE HEATING		
	a	*b*	*c*	*Ave.*	*a*	*b*	*Ave.*
612[c]	240	260	—	250	247	236	242
613[d]	546	560	—	553	506	526	516
614[d]	1000	1126	1078	1071	1008	950	979
615[d]	460	530	549	500	584	560	572
616[d]	428	440	510	473	548	578	563

[a] Mixed with CaO, 1:1, prior to heating at 500°C.
[b] As in footnotes of Tables 1 and 2 for soils and distillations.
[c] No input of $BaSiF_6$ in 1930.
[d] Input of $BaSiF_6$ in 1930, as in Tables 1 and 2.

In every case the fluorine content indicated by the 1935 analyses, and also by the 1938 analyses, was considerably less than the content that was registered by the 1949 analyses. In the 1935 analyses, the charges were subjected to calcination with calcium carbonate, but the distillation technique was not recorded, whereas, in the 1938 analyses the charges of soil were mixed with calcium peroxide and calcined prior to steam-current distillation from perchloric acid.

The 1949 findings obtained by means of the techniques (e), (f), (g) and (h) indicate relatively equal values for the native supply of fluorine for the limed and unlimed charges and likewise for the variations in the duration of calcination. In contrast, smaller recoveries of the fluorine inputs were registered by the distillates from the limed charges of high fluorine content that were subjected to the longer calcination.

The computed values under (a) represent native content of fluorine plus the respective inputs. Obviously, these values are higher than those of actual residues, since fluorine outgo was not determined during the three years they were exposed to rain-water leachings. The amounts of

fluorine leached during the fourth year were determined, however, and these are recorded in the footnotes of Table 4. Upon postulation of like leachings every year, the 4-year amounts to be deducted from the p.p.m. values given in column (a) would be 5, 400, 24, and 27 p.p.m. for soils 613, 614, 615, and 616, respectively. When appropriate subtractions of those quantities are applied to the several computed values given in column (a), the residues of fluorine then became 545, 1012, 526, and 523 p.p.m. against the respective determinations of 553, 1071, 500, and 473 p.p.m. that are given in column (g). Therefore, it appears probable that

TABLE 4.—*Fluorine content of soils that have received $BaSiF_2$ in 1930*

SOIL NO.	$BaSiF_6$ INCORPORATIONS PER 2,000,000 LBS. OF SOIL	COMPUTED FLUORINE CONTENT		FLUORINE DETERMINATIONS MADE						
		INPUT	TOTAL[a]	IN 1935[b]	IN 1938[c]	IN 1949[d]				
						e	f	g	h	i
		p.p.m.	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*	*p.p.m.*
612	None	0	246	96	132	246	256	250	242	276
613	Single, 1500 lbs.	304	550	288	372	426	545	553	516	381
614	Four, 1500 lbs. annually	1216	1412	896	743	1047	1086	1071	979	875
615	Single, 1500 lbs. plus limestone	304	550	404	472	486	476	500	538	395
616	Single, 1500 lbs. plus dolomite	304	550	196	402	477	522	473	537	337

[a] Computed as sum of content of the control in 1949 and amount of the fluorine input of 1930.
[b] Calcium carbonate used as fixative.
[c] Calcium peroxide used as fixative.
[d] Distillation from H_2SO_4 at 165°C. and from $HClO_4$ at 130°C. on evaporated aliquot.
[e] From unheated soil.
[f] From soil and CaO, 1:0.7, and heating @ 500°C., 30 minutes.
[g] From soil and CaO, 1:1, and heating @ 500°C., 5 minutes.
[h] From soil and CaO, 1:1, and heating @ 500°C., 60 minutes.
[i] From soil and CaO, 1:3, and heating @ 500°C., 30 minutes.

Fluorine leachings from the several soils during the 4th year of the lysimeter experiment as accounted for by the 1935 analyses of the leachates, were:

Nos.	*p.p.m. of F soil basis*
612	1.24
613	8.35
614	99.89
615	5.90
616	6.64

the double distillations of 1949 register complete recoveries of residual fluorine from the unlimed and the lightly-limed charges, whereas uncertain recoveries were obtained from those subjected to extended calcination and from the heavily limed charges.

Recoveries of fluorine from inputs of sodium fluoride. Since incorporations of sodium fluoride have been made to soils in related comparisons at this Station and in experiments conducted elsewhere, it seemed necessary to determine whether recoveries from such incorporations could be obtained from limed-soil calcines by means of double distillations. Since the incorporated fluoride of sodium undergoes conversion to calcium fluoride in soils of normal calcium content, that transition was assured for the

calcines of the charges that received sodium fluoride. The results of Table 5 register complete recovery of fluorine from the 100-p.p.m. inputs carried by soil-lime charges that were calcined 30 minutes at 500°C., whereas the recoveries from the 200 p.p.m. and 500 p.p.m. inputs were significantly incomplete, as the consequence of either partial retention or volatilization of the additive fluorine.

Fixation vs. volatilization as causes of incomplete recovery of additive fluorine. The liming of soil charges prior to their calcination has been

TABLE 5.—*Recoveries of fluorine through double distillation of ignited limed charges of a silt loam plus sodium fluoride*[a]

FLUORINE ADDITION	FLUORINE			
	FOUND		RECOVERY	
microgram	*p.p.m.*	*ave. p.p.m.*	*micrograms*	*per cent*
None[b]	100			
None[b]	120	110	—	—
None	120			
None	124			
None	124	122	—	—
100	220			
	222			
	226	223[c]	101	101.00
250	340			
	340			
	340	340[c]	218	87.20
500	604			
	564			
	560	576[c]	454	90.80

[a] The distillations were as in footnote (a) of Table 1 on charges or soil that were heated 30 minutes at 500°C after inmixing of 1 part of CaO into one part of soil.
[b] Without CaO.
[c] Sum of the 122 p.p.m. average and the amount added as NaF.

deemed essential to assure that content of native and additive fluorine then would be in state to preclude loss of that element during incineration, and yet assure complete volatilization under distillation. Although calcinations of the analytical charges may be imperative when perchloric acid distillations are made directly from highly organic materials, the prior ignition of rock-derived soils appears to be unnecessary, and may be inadvisable, for the direct distillation from sulfuric acid, the resultant distillates being caught in sodium hydroxide solution and then concentrated for distillation from perchloric acid. In the use of sulfuric acid for the distillation of fluorine from plant material, however, it is necessary that the analytical charges be slurried with calcium hydroxide and incinerated to

obviate the passage of sulfur dioxide and other volatiles into the distillates. This may be proved true also for mucks and for soils of high content of organic matter.

Obviously, disparities between actual contents of fluorine and the recoveries in the distillates from the calcined mixtures of soil and lime are attributable to either the fixation of fluorine in soil complexes more resistant than calcium fluoride against distillation from sulfuric acid or to

TABLE 6.—*Fluorine recoveries through "double" distillation[a] from 0.1-gram charge of CaF_2, limed and unlimed, that had been heated 30 minutes, alone and with quartz, and with soil, at 500°C. and at 900°C.*

CALCINATION OF THE CaF_2 CHARGE	FLUORINE RECOVERIES FROM CALCINES			
	WITHOUT CaO		WITH CaO	
	mgm	*disparity, per cent*	*mgm*	*disparity, per cent*
None	48.65	—	—	—
At 500°C.	45.91	5.83	47.19	3.00[d]
At 900°C.	44.22	9.91	46.75	3.95
At 500°C., with quartz[b]	48.83	0	42.33	12.99
At 900°C., with quartz[b]	40.23	17.31	29.76	38.83
At 500° C., with soil[b]	48.67	0	43.21	11.18
At 900°C., with soil[b]	38.43	21.01	29.59	39.18
At 500°C., with wetted soil[b,c]	38.86	20.12	45.72	6.02
At 900°C., with wetted soil[b,c]	27.34	43.80	30.16	38.01
Soil alone, at 500°C.	0.300	—	—	—
Soil alone, at 900°C.	0.182	—	—	—

[a] By means of H_2SO_4 on the charge and $HClO_4$ on the concentrated distillate.
[b] Equal parts.
[c] The dry mixtures were wetted and evaporated to dryness twice before they were heated.
[d] The loss of fluorine from a fluorspar parallel at 500°C. was found to be 1.46 per cent.

the volatilization of that fluoride, *per se*, or to its reduction, and possibly to a combination of those phenomena. Therefore, the fluorine determinations in Table 6 were made to ascertain whether calcium fluoride suffers a loss of fluorine when an analytical charge is calcined alone and in association with siliceous materials. Calcined 30 minutes at 500°C. and at 900°C., the 0.1-gram charges of dried c.p. calcium fluoride suffered fluorine losses of 5.83% and 9.91%, respectively. When like charges of calcium fluoride were mixed with quartz and with soil and calcined at 900°C., the respective losses of fluorine were 17.31% and 21.01%. When like mixtures of calcium fluoride and soil were wetted and dried twice and then calcined at 900°C., the 20.21 per cent fluorine loss was enhanced to one of 43.8 per cent.

Apparently, volatilization induced the substantial disparities between the fluorine of the calcium fluoride charges and the recoveries from their calcines, with and without inclusions of siliceous materials. An alternative postulation was that calcination induced the formation of solid fluoric complexes that yielded no fluorine during the sulfuric acid distillations. Since calcination of soil charges at 900°C. had been found to inhibit distillation-recovery of fluorine from additive fluorides (2), the fixation of the element in the calcines then had seemed to be the plausible supposition. Hence, the experiment of Table 7 was conducted to determine which

TABLE 7.—*Fluorine losses induced when 1-gram charges of calcium fluoride, and of fluorspar, were calcined 30 minutes at 900°C.*

CALCINES[a]	WEIGHTS		FLUORINE CONTENT[c]		DISPARITY IN FLUORINE RECOVERY AFTER HEATING	
	INITIAL	AFTER HEATING	INITIAL[d]	AFTER HEATING[e]	ACTUAL	PROPORTIONAL
	gm	*gm*	*mgm*	*mgm*	*mgm*	*per cent*
Fluorspar, alone	1.0000	0.9667	46.32	39.98	6.34	13.69
Fluorspar+CaO	2.0000	1.9495	24.80	23.83	0.97	3.91
CaF_2, alone	1.0000	0.9436	51.57	43.37	8.20	15.90
CaF_2+CaO	2.0000	1.8732	26.97	25.27	1.70	6.30
CaF_2+Quartz	2.0000	1.8821	26.57	17.67	8.90	33.50
CaF_2+Soil	2.0000	1.7943	27.87	19.53	8.34	29.92
CaF_2+Soil (wetted)[b]	2.0000	1.8013	27.76	17.37	10.39	37.43

[a] Charge of 1 gm of c.p. precipitated CaF_2 alone or with 1 gm of the added material.
[b] Wetted and dried, twice.
[c] Analytical charge of 0.1 gm.
[d] Calculated to allow for the diminution in charge-weight, as the result of the calcination of the full charge, and upon assumption that no loss of fluorine occurred then.
[e] Represents virtually the absolute quantities of fluorine present in the 0.1-gm analytical charges of the calcine that were subjected to H_2SO_4 and $HClO_4$ distillations, since fluorine was virtually nil in the distillates obtained when the solid residues from the H_2SO_4 distillations were dried and subjected to fusions and to $HClO_4$ distillations therefrom.

of the two possibilities—volatilization or fixation—was causal for incompleteness in the recoveries of fluorine. Because of the magnitude of the disparities between fluorine content and fluorine recovery, for the calcines obtained at 900°C. (Table 6), that temperature was imposed to afford better opportunity to determine whether the disparities were due in part to molecular volatilization of the fluoride or solely to its thermally-induced disintegration. The milligram values for fluorine content under "initial" were computed upon basis of the fluorine content of the calcium fluoride and of the fluorspar, and upon assumption that such content was yet present in the respective calcines.

The inclusion of lime in the charges of calcium fluoride and of fluorspar, induced a substantial lessening of the losses of fluorine from the calcines

of the two fluorides. In contrast, the inclusion of quartz in the analytical charge of the fluoride caused an increase of more than two-fold in the loss of fluorine. Similar effect resulted from inclusion of dry soil in the charge of calcium fluoride, whereas maximal loss of fluorine was registered by the corresponding mixture that was wetted and dried twice prior to its calcination.

Although the foregoing determinations indicate that the substantial losses of fluorine occurred as a result of volatilization at 900°C., there remains the possibility that the sulfuric acid distillations did not effect absolute removal of fluorine from the calcines. Therefore, the residual solids

TABLE 8.—*Decrease in calcium content of 1-gram charges of CaF_2 after they were heated 30 minutes at 900°C.*

CALCIUM FLUORIDE	WEIGHT OF CHARGE		CALCIUM CONTENT		CALCIUM DECREASES	
	INITIAL	AFTER HEATING	INITIAL	AFTER HEATING	ACTUAL	PROPORTION
	gm	*gm*	*mgm*	*mgm*	*mgm*	*per cent*
Alone	1.0000	0.9436	272.15[b]	249.5	22.65	8.32
Plus SiO_2[a]	2.0000	1.8821	265.70[c]	257.0	8.70	3.27

[a] Charge of 1 gm CaF_2 plus 1 gm of quartz.
[b] Analytical charge of 0.5-gm: its "initial" content of Ca was computed upon assumption of no loss of Ca during calcination.
[c] Analytical charge of 1 gm, with assumption as in (b).

from the sulfuric acid distillations reported in Table 7 were subjected to alkaline carbonate fusion and the resultant melts were pulverized and subjected to double distillation. The perchloric acid distillates from the concentrated sulfuric acid distillates were found to be virtually devoid of fluorine. Hence, volatilization, rather than fixation, is established as being responsible for the disparities between the quantities of fluorine carried by inputs of fluorides and recoveries of fluorine therefrom.

A further consideration was whether the disparities in the fluorine recoveries from the calcines were due in minor measure to volatilizations of calcium fluoride, as such. In that case, the residues (Table 7) from the 500°C. and 900°C. calcinations of calcium fluoride alone and with quartz, should register a decrease in calcium content. The findings given in Table 8 indicate that the calcium fluoride calcine suffered a calcium loss of 8.32 per cent, against a calcium loss of only 3.27 per cent from the calcined mixture of calcium fluoride and quartz. Therefore, it is obvious that, when calcium fluoride was calcined alone at 900°C., a fraction of the resultant loss of fluorine was due to a molecular volatilization. Apparently, the inmixed quartz served to induce the formation of calcium silicate in the calcine and thereby diminish the loss of calcium therefrom, in contrast to the accelerative effect that the quartz exerted upon volatilization of fluorine, as in Tables 6 and 7.

MAGNESIUM VERSUS CALCIUM IN THE CALCINATION OF SOIL CHARGES

In an earlier comparison of the efficacy of calcium peroxide and of magnesium peroxide, in the prior ignition of soil charges to be used for perchloric acid distillations, it had appeared that the input of magnesium served to diminish the recovery of additive fluorine (1). Since dolomite had been incorporated at a rational rate in soil No. 616 of Tables 1, 2 ,and 3, the recoveries of fluorine therefrom could be compared with the recoveries from the limestoned soil No. 615. The findings do not indicate that the magnesium of the moderate incorporation of dolomite induced a decrease in the double distillation recovery of additive fluorine, in contrast to the effects that were induced by the admixed peroxide of magnesium in the charges that were calcined and then subjected to direct distillation from perchloric acid (1).

SUMMARY AND CONCLUSIONS

Through fluorine determinations on certain fluoride-fortified soils, by means of the double distillation procedure, perchloric acid at 165°C. one hour; 250 ml. distillate, concentration thereof and perchloric acid distillation at 130°C., one hour and 200 ml. distillate the following conclusions were derived.

(a) Pre-calcination was unnecessary for the analytical charges of the soils used.

(b) Recovery of additive fluorine was not diminished by the premixing of lime in soil charges in equal proportion, whereas larger proportions of lime diminished such recovery.

(c) Protracted calcination of the limed charges lessened recoveries of fluorine from additives.

(d) Calcination of calcium fluoride, alone, at 500°C, and at 900°C. caused decreases in the recoveries of fluorine.

(e) Inclusions of quartz and of soil in the charges of calcium fluoride caused substantial decreases in the recoveries of fluorine from the calcines.

(f) The volatilization of fluorine from the 900°C. calcines of calcium fluoride and inmixtures of lime was lessened, through protective formation of calcium silicate.

(g) The 900°C. calcinations of 1-gram charges of calcium fluoride, alone and with quartz, induced calcium losses of 8 per cent and 3 per cent, respectively, which registered minor volatilization as calcium fluoride and major losses of fluorine in gaseous phase.

(h) Like recoveries of fluorine were obtained from soils, previously limestoned and dolomited, that had received substantial inputs of barium silicofluoride in a lysimeter experiment.

The findings demonstrated that the disparities between the fluorine content of the fluoride-fortified soils and recoveries from their calcines

were due to volatilization of fluorine and not to its fixation in the calcines. It is concluded, also, that for soils of the type used, the direct double distillation procedure is preferable to the procedure whereby the soil charge is calcined, with or without the inclusion of lime, prior to the step or steps of distillation.

The findings serve to demonstrate the importance of additional studies that would include samples of high content of organic matter in testing further the efficacy of the double distillation procedure on raw soils, and on calcines of their limed charges.

ADDENDUM

Subsequent to the presentation of this paper, the authors received "Industrial Fluorosis" A Study of the Hazard to Man and Animals near Fort William, Scotland, Medican Research Council Memorandum No. 22, His Majesty's Stationery Office (London), 1949. In that report it was stated that Solochrome Brilliant Blue B.S.—the sodium salt of sulphodichloro-hydroxy-dimethyl-fuchsin dicarboxylic acid—had been found superior to the alizarinsulfonate indicator in the thorium nitrate titrations.

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REPORT ON EXCHANGEABLE POTASSIUM OF SOILS

By A. MEHLICH (North Carolina State College of Agriculture and Engineering, Raleigh. N. C.), *Associate Referee*

Collaborative study on exchangeable potassium in the course of the past two years have shown the cobaltinitrite and flame photometer procedures equally well adapted. Following the precipitation of potassium with sodium cobaltinitrite the potassium is determined volumetrically with ceric sulfate or colorimetrically with Nitroso-R-Salt. The volumetric procedure is usually not very accurate with potassium concentrations less than 1 p.p.m., whereas the Nitroso-R-Salt procedure is particularly well adapted to much lower levels and works well over a wide range of potassium concentrations. The cobaltinitrite procedure is relatively time consuming and requires carefully controlled techniques. In contrast, the flame photometer procedure is rapid and convenient. With lithium as the

internal standard, the potassium can be determined directly in the soil extract without apparently the need for removing excess salts. Further collaborative studies with the cobaltinitrite and flame photometer procedures are now in progress.

While there are several procedures which seem to work satisfac'orily after the potassium has been extracted from the soil, there is little evidence of agreement as to the best method for securing the extract. Work has now been initiated to study some of these problems, such as nature and concentrations of salts, soil-solution ratio, extraction techniques, etc. It is for the present not advisable, therefore, to recommend a definite procedure. On the basis of the 1948 report by Dr. Miles, the procedures, published in: U.S.D.A. Circular 757, 1947; Soil Science, 67: 123–136, 1949; Soil Science 67: 439–445, 1949; Soil Science 66: 429–445, 1948; and Soil Science Society of America Proceedings, 11: 221–226, 1947, have yielded fairly satisfactory results. It appears likely that ultimately several alternate procedures can be used successfully.

A paper entitled "Factors to be Considered in the Development of Methods for Determining Cation-Exchange Properties," by A. Mehlich, was presented at the meeting.

An address "Possible Contributions to Food and Drug Problems by the Atomic Energy Program," was given by L. W. Tuttle.

REPORT ON PRESERVATIVES AND ARTIFICIAL SWEETENERS

By MARGARETHE OAKLEY (State Department of Health, Baltimore 18, Md.), *Referee*

The chapter on Preservatives and Artificial Sweeteners is a very old one in years and antiquity of its methods. It has been the aim of this Referee to modernize it. That could not be done in a year, but after several years of groundwork the accomplishments of the Associate Referees for 1949 have been most gratifying. If the recommendations for changes in the chapter are acceptable to Committee C, over half of the plans for bringing it up to date will have been completed.

RECOMMENDATIONS*

In line with this policy the Referee recommends the deletion of the methods for Abrastol and B-Naphthol. As far as can be ascertained by wide-spread questioning over the past two years, these two compounds

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 55 (1950).

are not being used at the present time; and although the methods need revision, it is thought advisable to delete them rather than have an Associate Referee work over methods for obsolete products.

Under the exigencies of the situation, it is recommended that the qualitative test for saccharin, 6th Edition **32:13**, known as the phenol sulfuric acid test, be made first action, based on the collaborative results reported in 1941.[1]

Also recommended for deletion are all seven of the methods for the detection of formaldehyde. These have been handed down from the first *Book of Methods*, and collaborative work has proved newer methods to be superior to them. It is, therefore, recommended that the chromotropic acid test and the Hehner-Fulton test for formaldehyde be adopted, first action, in place of the seven to be deleted.

The qualitative tests for dulcin have been under fire for several years. The work just completed by the Associate Referee justifies recommendation of their deletion and the substitution of the Denigès-Tourrou test and the modified La Parola-Mariani test for dulcin under the "first action" classification.

The sublimation method for the determination of saccharin was subjected to collaborative study and is recommended for adoption as first action.

It is recommended that the method for formic acid in the chapter on preservatives and artificial sweeteners be assigned an Associate Referee for the purpose of bringing this part of the chapter up to date.

It is recommended that an Associate Referee be appointed to study the qualitative tests for fluorides. The references to the methods, now official, are dated 1905 and 1895, and the tests have been criticized by A. O. Gettler and L. Ellerbrook[2] as lacking sensitivity.

The method for thiourea in oranges and orange juice identified as the Rapid Oxidation Method was adopted last year as tentative. Collaborative work was done this year and the method with a slight change in directions is recommended for adoption as first action.

Although the method for "Volatile Fatty Acids in Bakery Products" was adopted last year, the Associate Referee on Mold Inhibitors continued his studies, and a slight change in wording in the method is recommended. This is for the purpose of clarification in the directions for calculations. His new investigations were on a rapid distillation procedure and a chromatographic separation of the acids. He expects to submit his findings to collaborative study and to continue his investigations on methods of preserving bread samples against spoilage and loss of volatile acids during storage.

Further plans for the coming year include study of a method for the

[1] *This Journal*, **24**, 327 (1941).
[2] *Am. J. of the Med. Sciences*, **19**, 625–638: 1939.

determination of dulcin and a method for the detection of 1-propoxy-2-amino-4-nitrobenzene, by the Associate Referee on artificial sweeteners.

Although many deletions are recommended for this chapter in the Seventh Ed., of *Methods of Anlaysis*, they are effectively counterbalanced by much needed methods for the newer preservatives of current interest. The Referee is greatly appreciative of the tremendous amount of work done by the Associate Referees in accomplishing this renovation, and concurs in their individual recommendations.

REPORT ON QUATERNARY AMMONIUM COMPOUNDS IN FOODS (INCLUDING EGGS)

By JOHN B. WILSON (Food and Drug Administration, Federal Security Agency, Washington 25, D.C.), *Associate Referee*

The Associate Referee is not yet ready to report on the collaborative samples containing quaternary ammonium compounds which were sent out in the summer of 1948 because during the past year it was discovered that two new food products were being subjected to treatment with these compounds and his immediate attention was required to provide methods for the analysis of these foods (eggs and shrimp).

The first of these products, frozen eggs, are liable to contain quaternary ammonium compounds through accidental contamination, due to the use of these compounds as sanitizing and cleaning agents in egg-breaking establishments, as well as by intentional addition of them for their bactericidal effect.

QUATERNARY AMMONIUM COMPOUNDS IN EGGS[1]

During the past few years it has become a common practice to use solutions of quaternary ammonium compounds as cleansing and sanitizing agents in egg breaking establishments. It has also been rumored that these chemicals have been added to frozen eggs as preservatives and deodorizers. As only small quantities of quaternary ammonium compounds would be incorporated into the final product, either by inadequate drainage or the non-rinsing of the equipment or the cans used, or even in the case of deliberate addition as preservative, the application of the bromophenol blue method was studied.

No means has been found of extracting quaternary ammonium compounds directly from foods. It has always been necessary in cases of solid foods to get the quaternary ammonium compounds in solution with ingredients of the food that dissolve, and then to remove the food constituents by any means possible and make the test on the final solution containing the quaternary in as pure a state as possible to obtain.

It was found that when the egg was mixed with acetone, which is a

[1] Contributions from Division of Food, W. B. White, Chief.

good solvent for quaternary ammonium compounds, the fatty matter, including lecithin, phosphotides, etc., dissolved, leaving the protein in the form of a powdery precipitate which was filtered off. After filtration, the acetone solution was diluted with water, causing much of the fatty matter, etc., to separate out in the form of an oily layer on top of the mixture. This was removed by extraction with petroleum ether. At this point the analyst must be very careful not to agitate the mixture too strenuously; otherwise an emulsion will form which is very difficult to

TABLE 1.—*Quaternary ammonium compounds in eggs*

ADDED, PER 100 G EGG	FOUND, PER 100 G EGG			
	WILSON		FELMAN	
	mg	*per cent*	*mg*	*per cent*
1 mg Z[1]	0.44	44	0.23	23
	0.57	57		
1 mg DC 12[2]	0.52	52		
	0.55	55		
	0.55	55		
2 mg Z	0.92	46	0.65	33
	1.00	50		
	0.94	47		
2 mg DC 12	1.20	60	1.40	70
	1.27	64		
	1.15	58		
4.76 mg Z	3.40	71.4	2.00	41
	3.36	70.6		
3.92 mg DC 12	3.10	79	2.60	67
	2.14	55		

[1] Alkyldimethylbenzylammonium compound (mol. wt. 357).
[2] Lauryldimethylbenzylammonium chloride.

handle. The lecithin and other compounds are such efficient emulsifiers that any emulsion formed at this point will take a long time to separate. Even after all of the yellow color of the fat has disappeared from the acetone water layer, the petroleum ether layer will have a noticeable color. The writer has noticed a tendency toward emulsification even when the petroleum ether layer has a very pale yellow color. Since any of these emulsifiers will cause difficulty when the ethylene chloride extraction is made, they must be removed as completely as possible.

The writer recommends that, if the four extractions required in the method do not remove all color, the analyst continue extracting until the petroleum ether remains colorless. When making the fourth extraction, the separatory funnel can usually be shaken quite vigorously without form-

ing an emulsion. The method in detail is given in *Methods of Analysis*, 7th Ed., 1950.

To test out the method, several samples of frozen eggs were prepared as follows: Place the eggs in a beaker and mix thoroughly with a soda-fountain milk shake mixer. Weigh 100 grams of egg into a 250 ml. beaker and again mix as above. While the mixer is running, add with a pipet 1 to 5 ml. of standard quaternary ammonium compound solution as desired, and continue mixing several minutes. Pour the mixture into a wide mouthed bottle and place in the deep freeze.

In preparing the samples, two quaternary ammonium compounds were used, namely, lauryldimethylbenzylammonium chloride and alkyldimethylbenzylammonium compound (mol. wt. 357). The results by two analysts are given in Table 1.

While the recoveries in Table 1 are not as high as desired, they become greater with the analyst's familiarity with the method and are about as great as would be expected when we consider the opportunities for loss in carrying out the procedure. Collaborative results are reported below.

The method for eggs finally devised is presented in *Methods of Analysis*, 7th Ed., 1950. Discussion of the development of the method is given below.

In preparing the samples for collaborative study frozen eggs known to be free from addition of quaternary ammonium compounds were first

TABLE 2.—*Quaternary ammonium compounds in frozen eggs*
(p.p.m.)

COLLABORATOR	SAMPLE		
	A	B	C
Added DC12[1]	10	20	50
Found			
Matthew L. Dow		16.0	40.5
		18.8	46.9
Harold Felman	5.5	13.1	22.1[2]
	5.5	13.2	30.0[2]
Jacob Marder	6.8	18.2	43.1
	7.3	17.4	38.7
John B. Wilson	6.0	16.5	46.0
	6.3	15.5	44.0
Average	6.2	16.1	43.3
Recovery	62	80	87

[1] Lauryldimethylbensylammonium chloride.
[2] Omitted from average recovery.

analyzed by the method. Portions of the frozen egg were then melted, the melted egg was thoroughly mixed, using a milk shake mixer of the type used at most soda fountains, and while the mixer was going a small quantity of a water solution (not more than 1% the weight of egg used) containing the requisite quantity of quaternary ammonium compound was added by means of a pipet. The mixer was allowed to run for 2–3 minutes after this addition.

The results obtained are given in Table 2.

As a precaution against non-uniformity of reagents, which may be responsible for variations in previous results with the bromophenol blue method, all collaborators were furnished quantities of DC12 to be used in making the standard curves, bromophenol blue from the same batch, and anhydrous sodium sulfate from the same shipment.

The results in Table 2 warrant the adoption of the proposed method for frozen eggs as first action.

SUMMARY

A method is given for the determination of quaternary ammonium compounds in eggs, and results by two analysts reporting on the analysis of several samples of eggs to which known amounts of two quaternary ammonium compounds were added.

The other food for which it was found necessary to provide a method for quaternary ammonium compounds was shrimp. Certain manufacturers had immersed shrimp in solutions of quaternary ammonium compounds for cleaning purpose, unaware of the fact that these creatures absorb more or less of these compounds in a manner very difficult or perhaps impossible of removal. Later the shrimp were washed extensively with water, then frozen and shipped into U. S., where they were detained when it was found that they contained some of this poisonous preservative.

While the method itself has reached a stage where publication may be warranted, there appear to be still some problems regarding sampling, etc., which must be overcome before collaborative results obtained this year can be given out.

RECOMMENDATIONS*

It is recommended.—

(1) That the method for quaternary ammonium compounds in eggs described in this report be adopted as first action.

(2) That collaborative study be continued on the following methods:

(a) Method for Fruit Juices, *This Journal*, **29**, 318 (1946).

(b) Shorter Method for Fruit Juices, *Ibid.*, **29**, 319 (1946)

(c) Method for Bottled Sodas, *Ibid.*, **29**, 323, subject to increasing the volume of bromophenol blue reagent to 5–10 ml.

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 55 (1950).

(d) Method for Milk, *Ibid.*, **29**, 324, on samples containing preservative quantities of quaternary ammonium compounds.

(e) Method for Mayonnaise, Salad Dressings, and Sandwich Spreads, *Ibid.*, **29**, 323 (1946).

(f) Method for Pickles and Relishes, *Ibid.*, **29**, 326 (1946).

(3) That work be continued on the determination of quaternary ammonium compounds in shrimp.

REPORT ON QUATERNARY AMMONIUM COMPOUNDS IN SHRIMP

By JOHN B. WILSON (Chemist, Division of Food,[1] Food and Drug Administration, Federal Security Agency, Washington 25, D.C.), *Associate Referee*

In the fall of 1948 it was found that certain lots of shrimp imported to the United States contained quaternary ammonium compounds. These shrimp had been washed in solutions of alkyldimethylbenzylammonium chloride, then rinsed with water, frozen in 5-lb. blocks, and glazed with ice.

As in previous work on foods[2] it was found expedient to get the quaternary ammonium compound into solution, remove as much other material from the solution as possible, concentrate it, and determine the quaternary ammonium compound in the residual liquid by the bromophenol blue method.

The method developed for shrimp after considerable experimentation follows:

METHOD

APPARATUS

Waring Blendor.

Variable voltage transformer (or rheostat) to regulate speed of blendor.

Büchner funnels (like Pyrex No. 36060, medium porosity (a) 60 mm disc, 150 ml capacity; (b) 90 mm disc, 600 ml capacity.

Separatory funnels (a) 1 liter capacity; (b) 2 liters capacity.

REAGENT

Bromophenol blue; solid tetrabromophenol sulfonphthalein (must give clear blue color having maximum absorption at about 608 millimicrons when treated as under "Preparation of Standard Curve").[3]

SOLVENTS

Acetone.—A. C. S. Reagent.

Petroleum ether.—A. C. S. Reagent.

Ethylene Chloride., Eastman Kodak Co.—Practical grade or equivalent.

NOTE: When determining quaternary ammonium compounds in foods, all glass-

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[1] W. B. White, Chief.
[2] *This Journal*, **29**, 312 (1940).
[3] See spectrophotometric curve made from satisfactory reagent (Eastman Kodak Co.), *This Journal*, **29**, 315 (1946).

ware must be scrupulously clean. Soap is especially to be avoided since interaction occurs between the two classes of compounds. For this reason, if soap is used in cleansing, all glassware should be rinsed thoroly with water. As an extra precaution, it has been found advisable to rinse all pipets with alcohol and dry by suction before use.

PREPARATION OF SAMPLE

Obtain one or more blocks of frozen shrimp, thaw and drain off water. Mix the shrimp and arrange on a flat surface in a single layer in the form of a disc. Select individual shrimp from different locations in the disc in such a manner as to be as representative as possible of the whole, until a quantity weighing 750–1000 g is obtained. Separate the shells from the flesh of these shrimp, weigh, and calculate the percentage of shell and flesh in the whole shrimp. Weigh out two separate 200-g samples of the flesh and two separate 50-g samples of the shell, making all samples as representative as possible, and proceed as directed.

PROCEDURE

Place each sample in Waring blendor, add 100 ml of acetone, and blend for 1–2 min., regulating the speed of the blendor so that none of the mixture will be thrown out of the jar during the blending. Transfer the mixture to a 400-ml beaker, using ca 100 ml of acetone to wash out the jar in the case of the flesh, and ca 50 ml for the shell.

Let stand for ca 1 hour, stirring occasionally with a glass rod. Filter, using for the shell a Büchner funnel (a) (60 mm), and for the flesh a Büchner funnel (b) (90 mm). In the case of the flesh, keep the filtration moving by scraping up the cake from the fritted disc with a glass rod.

After the liquid has been drawn off, turn off the suction, add 50 ml acetone to the shell and 100 ml to the flesh. Mix carefully with a glass rod so that the entire sample is contacted by the liquid, let stand 5 min. and draw the liquid thru with suction. Repeat until most of the pink coloring matter is removed from the sample. (At least three washings should be made.) Mark the level of the liquid in the suction flask and transfer the liquid to a large separatory funnel. (Use the 1 l funnel for the extract of shell and the 2 l funnel for the flesh.) Wash out each suction flask with 25 ml of acetone. Fill each suction flask with water to the level marked for the filtrate and add to the proper separatory funnel. Add 25 ml HCl to the separatory funnel containing the shell extract and 35 ml to the one containing the extract of the flesh, and mix. Add 300 ml of petroleum ether to each suction flash; agitate to dissolve any colored substances which still adhere to the sides of the flask and add to their respective separatory funnels.

Give the suction flasks a rinse with 25 ml acetone and finally with 25 ml of water, adding to their respective funnels. In the case of the flesh extract, add an additional 100 ml portion of petroleum ether at this point. Shake the separatory funnels gently by inverting and mixing slowly with a rotary motion so as to avoid the formation of any emulsion.

Let stand until the top layer is clear, draw off the lower layer into a 1 l beaker for the shell, and a 1.5 l beaker for the flesh. Be sure to draw off any emulsion into the water layer, as quaternary ammonium compound may be present in this fraction. Discard the petroleum ether layer.

Continue to extract carefully, using 300 ml portions of petroleum ether until the top layer is colorless, then make one additional extraction using 200 ml of petroleum ether. As the extraction progresses, the separatory funnel may be shaken more vigorously with less danger of producing an emulsion. However, at all times it is important that all emulsion-like material at the interface be allocated to the water layer.

When the extraction is complete, add 25 to 30 ml of water to each (now empty)

separatory funnel, shake, and add to the samples in the beakers. Repeat with 25–30 ml of alcohol.

Add a few glass beads and evaporate the acetone water solns on the steam bath until all odor of acetone has disappeared and the volume has been reduced to 75–100 ml. Remove from the steam bath; while still warm, add 10–20 mg of bromophenol blue to each, rotate the beakers to dissolve and to wet the sides, then wash down the sides of beakers with water. When cooled to room temp., pipet 50 ml of ethylene chloride into the beaker, allowing the liquid to flow down the sides of the beaker. Transfer to a separatory funnel, using a little water to wash out the ethylene chloride. Shake for about 1 min. Return the liquid to the beaker by pouring down the sides of the beaker. Again return to separatory funnel and shake for 2 min. When the lower layer is reasonably clear, transfer to a second separatory funnel, add 10 ml of 1% Na_2CO_3 and shake carefully for ca two min. If the lower layer is blue, quaternary ammonium compounds are present.

If the blue color has a depth suitable for reading, draw off the lower layer into a 125 ml glass stoppered Erlenmeyer flask containing ca 2 grams of anhydrous Na_2SO_4. Let stand for one-half hour and read in the photometer as directed under "Preparation of Standard Curve." From the reading find the corresponding quaternary ammonium compound content from the standard curve.

If the color is too deep to read, acidify the liquid in the second separatory funnel with 3–5 ml of HCl, shake till yellow, and return to the first separatory funnel, using a little water for the transfer. Now pipet an additional 50 ml of ethylene chloride into the first separatory funnel, shake for 2 min., and let stand. When a good separation has occurred, draw off the lower layer thru a 7 cm filter paper (S & S 589 white ribbon or equivalent) into a 125 ml Erlenmeyer flask and stopper. (Soln A).

Find the proper aliquot as follows: Pipet 25 ml of ethylene chloride and 5 ml of soln A into a 125-ml separatory funnel, add 10 ml of 1% Na_2CO_3 and carefully shake for 2 min. Let separate and observe the lower layer. If the depth of color is suitable for reading, make up to 50 ml by adding 20 ml of ethylene chloride from a pipette, shake 1 min., let settle, and draw off the lower layer, dry and read as directed above.

If the color is not deep enough, add more of soln A in 5 ml increments until a suitable depth of color is obtained, add solvent if necessary to a total of 50 ml, shake, draw off, dry, and read as above.

When the proper aliquot has been found, check the reading as follows: Pipet the aliquot of soln A into a 50 ml volumetric flask, fill to the mark with ethylene chloride and pour into a 125 ml separatory funnel. Pipet 10 ml of 1% Na_2CO_3 soln into the volumetric flask, swirl, pour into the separatory funnel, and wash out the volumetric flask with 2–3 ml of H_2O from a wash bottle. Shake, settle, draw off, dry, and read as above, adding about 5 mg of dry bromophenol blue to the separatory funnel if the aliquot used was 10 ml or less.

Preparation of the Standard Curve: Weigh 100 mg of D. C. 12[1] dissolve in H_2O and make up to 100 ml. Dilute 5 ml of the stock soln to 500 ml with water. Pipet 5 ml of the stock soln to 500 ml with water. Pipet 5 ml, 10 ml, 20 ml and 25 ml of the dilute soln (1 ml = 0.01 mg D. C. 12) into four separatory funnels, add 5–10 mg of solid bromophenol blue and 1 ml of HCl (1+1) to each. Pipet 50 ml of ethylene chloride into each and shake for 2–3 min. When clear, draw off the lower layer into another separatory funnel, add 10 ml of 1% Na_2CO_3 soln, invert and shake carefully for 2–3 min. When clear, draw off each lower layer into a glass stoppered flask containing 1–3 g of granular anhydrous Na_2SO_4 and after 30 min. read in the neutral

[1] If this compound is not available, any solid quaternary ammonium compound of known composition may be used. If none such is available, prepare the standard solution from a commercial solution of quaternary ammonium compound which has been standardized by the ferricyanide method, *This Journal*, 31, 105 (1948).

wedge photometer using a 1 inch cell and a light filter centering at 610 mμ. Use the data to draw a curve.

In our work it has been found convenient to record the results in terms of mg. D. C. 12 per 100 g of flesh and shell respectively, and then to recalculate to the basis of mg per kg (p.p.m.) of the whole shrimp using the percentages of flesh and shell obtained in the preparation of sample.

In case it is desirable to use another type of color-measuring instrument, ascertain by use of the above procedure, the maximum and minimum concentrations of the quaternary ammonium compound which produces in 50 ml of ethylene chloride, colors of a density suited to the instrument, and prepare a set of three or more standards covering the range between these two points, to be used in preparing the curve.

In treating shrimp with quaternary ammonium compounds in the laboratory, the writer sought to follow the commercial procedure as closely as possible. Blocks of frozen shrimp were obtained and thawed. The shrimp were placed in a large glass jar, covered with a solution containing about 200 p.p.m. of D. C. 12 (lauryldimethylbenzylammonium chloride) for 45 minutes, stirring the mixture occasionally with a glass rod. At the appointed time the liquid was decanted, replaced with water, and again stirred with the glass rod. After standing 5 minutes the water was decanted. After four such washings the shrimp were drained as thoroughly as possible and sampled for analysis. The results are given in Table 1.

TABLE 1.—*Analysis of shrimp for quaternary ammonium compounds*

	WT. GRAMS	PER CENT OF WHOLE	D. C. 12 FOUND	
			MG/100 G SAMPLE	MG/KG SHRIMP
Shell	51	20.5	1.80	
	60	20.2	2.45	
Average		20.35	2.12	4.32
Flesh	198	79.5	0.66	
	237	79.8	0.70	
Average		79.65	0.68	5.42
Total				9.74

Unfortunately, we have found no way of estimating the per cent recovery by the method since we have no way of knowing the actual quantity of quaternary ammonium absorbed by the shrimp. However, the analyst can say definitely that the quantity present is at least as much as was found, and probably more.

In a previous experiment made during the development of the present procedure, 1 kg of shrimp was immersed in 1 liter of solution containing

192 mg of D. C. 12. A determination of quaternary, in the decanted liquid showed 87 mg of D. C. 12 remaining in solution, indicating the removal of 105 mg. of D. C. 12. Analysis of the shrimp showed 18 mg of D. C. 12 in the shrimp, which leaves 87 mg of D. C. 12 unaccounted for.

Two possibilities are open to account for this apparent loss, *i.e.* (1) absorption of D. C. 12 by the shrimp in a manner in which it was not extracted in the analysis; (2) precipitation in the liquid by soluble matters leached from the shrimp during the treatment. It is a well known fact that fatty acids, their salts and esters, react with quaternary ammonium compounds to form precipitates. In this case a considerable quantity of precipitate was observed in the decanted liquid. Several fruitless attempts were made to filter the precipitate with a view to analyzing it for quaternaries, but decomposition set in before this could be accomplished.

Several commercial samples of shrimp which had been treated with quaternary ammonium salts have been analyzed by the method. Quantities of alkyldimethylbenzylammonium chloride (mol. wt. 357) found varied from 4.9 mg to 92.6 mg per kilo of shrimp.

REPORT ON MONOCHLOROACETIC ACID

By JOHN B. WILSON, Food and Drug Administration, Federal Security Agency, Washington 25, D. C., *Associate Referee*

The Associate Referee prepared four samples of beverages for collaborative study this year and received reports from four of those to whom the samples were submitted.

Sample 11 consisted of orange juice prepared according to label directions from a commercial frozen concentrated orange juice; 1.86 grams of monochloroacetic acid was dissolved in a portion of the water added and the total volume was 10 liters. After filling the individual bottles of juice they were heated in a steam sterilizer for one hour with loosened caps. The caps were tightened as soon as the bottles were taken out of the sterilizer.

This sample was intended for quantitative as well as qualitative examination. The remaining samples were for qualitative test only and their composition is described below.

Sample 12. A case of beer was purchased and placed in a room kept at 0°C. until thoroughly chilled. A water solution of 2 grams of monochloroacetic acid in 250 ml was prepared. When the beer was cold, the bottles were opened and 5 ml of the monochloroacetic acid solution was pipetted into each. The bottles were recapped immediately and mixed. Each 12 fluid ounce bottle therefore contained 40 mg of chloroethanoic acid and the beer contained about 11 mg per 100 ml.

Sample 13. A case of 12 fluid ounce bottles of Cherry Soda was obtained from a local grocery and placed in the room at 0°C. to cool. A solu-

tion of monochloroacetic acid containing 1.5 g in 250 ml was prepared and 5 ml added to each bottle using the same technique as for the beer. In this case each bottle contained 30 mg of chloroethanoic acid and each 100 ml, about 8.5 mg of the preservative.

Sample 14. Two 1 gallon bottles of California Burgundy wine were purchased; 0.532 gram of chloroethanoic acid was dissolved in a portion of the wine and more wine added until a total volume of 3.8 liters was obtained. The wine therefore contained about 14 mg of chloroethanoic acid per 100 ml.

Collaborators were instructed to make duplicate determinations of monochloroacetic acid on 100 ml samples of the orange juice using continuous extractors as described in *This Journal*, **32**, 97 (1949), and extracting for the length of time found necessary for a recovery of at least 95% when known quantities of monochloroacetic acid are extracted in the apparatus.

They were also instructed to make duplicate determinations on 100 ml samples of the orange juice using the equally efficient means of extraction given in *This Journal*, **32**, 97, par. (2).

The final instructions were to apply the indigo test and pyridine test to all four samples as described. *loc. cit. p. 99.*

The results of the quantitative work are given in Table 1.

TABLE 1.—*Monochloroacetic acid in orange juice*
(mg per 100 ml)

COLLABORATOR	EXTRACTION	
	CONTINUOUS	HAND
Present	18.6	18.6
Found:		
E. H. Grant	15.1	13.5
	15.2	13.5
O. C. Kenworthy	15.2	10.7
	15.7	12.3
F. C. Minsker	14.0	6.0
	14.5	6.5
J. B. Wilson	16.1	13.2
	15.8	12.1
Average	15.2	11.0
Recovery %	81.7	59.1

All collaborators experienced difficulties with emulsions when using the separatory funnels for extraction. E. H. Grant believes that if an increased

quantity of ether (130 to 150 ml) should be used the emulsions would be less likely to occur. O. C. Kenworthy found no difficulty with emulsions on this sample when he used 150 ml of ether for each extraction.

Collaborators' results agree very well in the determinations using continuous extractors. The average recovery for this method was 81.7% this year with 18.6 mg present compared with 86.8% last year when 22.7 mg were present. We are not able to attribute the low recovery of 80 to 85% to decomposition of monochloroacetic acid in orange juice since previous experiments have shown that the quantity present suffers no diminution over a period of 384 days in one experiment (*This Journal*, **27**, 198), and 30 months in another (*loc. cit.*, p. 199), although recoveries were not complete when known amounts were added, *loc. cit.*, and *This Journal* **25**, 149.

The Referee is satisfied that orange juice is not a fit subject for extraction with separatory funnels. All collaborators have had difficulty with emulsions and the recoveries are too low (75.3% last year and 59.1% this year) to warrant further work on this project. The Referee believes that in the case of orange juice continuous extractors must be used, although they are admissible for use with carbonated beverages and beer, and seem to be preferable for the extraction of monochloroacetic acid in wine. (See last year's report *This Journal*, **32**, 493.)

Reporting on the qualitative tests, Mary C. Harrigan, O. C. Kenworthy, F. C. Minsker, and John B. Wilson, obtained positive tests on all samples with the pyridine test, although Mr. Minsker considers this test very long and tedious as well as inconclusive because of the danger of a precipitate due to impurities, which might be taken for a positive test.

O. C. Kenworthy, F. C. Minsker, and John B. Wilson reported positive results by the indigo test. Mr. Minsker found the indigo test weak but positive using 100 ml of samples 12 and 13, so he repeated the test using 200 ml of sample and obtained a very definite positive test. Miss Harrigan had no fusion block available and tried to make the fusion in a muffle furnace on one set, and with a micro burner on a second set of determinations, without success.

The Referee concludes that both qualitative tests should be made official at this time.

RECOMMENDATIONS*

It is recommended—

(1) That the method for determination of monochloroacetic acid, *This Journal*, **31**,104 (1948) be adopted as official for beer and wine.

(2) That the following parenthetical expression be added to the method for monochloroacetic acid in carbonated beverages, beer, and wine, *This Journal*, **31**,105, after the sentence ending "extract with ether 2–3 hours," ("Use the length of time found necessary for a recovery of at least

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 55 (1950).

95 per cent when known quantities of monochloroacetic acid are extracted in the apparatus.") (Official.).

(3) That the following method be adopted as official.

DETERMINATION OF MONOCHLORACETIC ACID IN COMMERCIAL PRESERVATIVES

Prepare a dilution of the sample that will permit the measurement of a convenient aliquot containing 50–100 mg of monochloroacetic acid and determine in such aliquot as directed under the method for carbonated beverages.

(4) That the following qualitative tests for monochloroacetic acid in commercial preservatives be adopted as official; *This Journal*, **32**, 97 (A) Barium Test, (B) Barium-Indigo Test, (C) Indigo Test, (D) Pyridine Test.

(5) That the qualitative tests for monochloroacetic acid in carbonated beverages, orange juice, beer and wine be adopted as official as given in *This Journal*, **32**, 99.

(6) That the parenthetical phrase "(Except in the case of orange juice)" be inserted after the statement "The following equally efficient means of extraction may be used" in the method for monochloroacetic acid.

(7) That the method for the determination of monochloroacetic acid *This Journal*, **31**, 104 be adopted as first action for orange juice.

(8) That further work be done on the determination of monochloroacetic acid in fruit juices other than orange juice.

REPORT ON MOLD INHIBITORS, PROPIONATES

By LEWIS H. MCROBERTS (U. S. Food and Drug Administration, Federal Security Agency, San Francisco, California), *Associate Referee*

DETERMINATION OF ACETIC AND PROPIONIC ACIDS IN BREAD

The previous reports on this subject have described a procedure for the determination of volatile fatty acids in bread (1) (2) and the estimation of losses of acetic and propionic acids during baking and during air drying of samples (3). It has been shown that while formic and acetic acids are normally present in small amounts, propionic acid is not a normal ingredient. It has been recommended that the Associate Refereeship be continued to investigate partition chromatographic separation as an alternative procedure. Distillates from the Hillig volatile acid apparatus, following the Hillig procedure, (4) or a more rapid distillation procedure for obtaining total acetic and propionic acids could be used as bases for final chromatographic determination.

During the present year experiments have been conducted on methods of sample preparation and treatment to insure against spoilage of bread and loss of acids.

A rapid distillation procedure followed by chromatographic separation

has been found to give results comparable to those obtained by the method previously described (2).

SAMPLE PREPARATION

The previously reported procedures (2) for reduction of loaves of bread to a finely divided condition have proved inadequate to insure against spoilage of fresh bread or partial loss of volatile acids during air drying. In that it may be necessary to store samples before or after analysis, methods of preservation should be developed. The following methods have been given preliminary trials: The prepared fresh bread (2) was treated with chloroform. About 5–10 ml of chloroform absorbed in 1 g. of cotton was added to ¾ quart of finely divided fresh bread in a closed Mason jar. The jar was rolled to distribute the chloroform vapor and stored at room temperature. This seems to be adequate to prevent mold formation. Further storage experiments will be made to detect any change in the volatile acid content.

Loss of volatile acids during air drying of samples might be prevented if the bread was made slightly alkaline just previous to the drying process. To accomplish this change the Associate Referee tried holding the sliced bread in a large desiccator over ammonia and followed this treatment with the usual air drying to a crisp condition and grinding to 20 mesh. The samples prepared in this manner were analyzed in comparison with the fresh bread and untreated air dried bread. The results on four commercial breads labeled in part "—Calcium Propionate added to retard spoilage—" are tabulated in Table 1.

TABLE 1.—*Propionic and acetic acids in commercial breads*
(Sample preparation comparison)

BREAD DESCRIPTION	ANALYSES (CALC. TO FRESH BASIS: MG/100 G)*					
	PREPARED FRESH BREAD		PREPARED AIR DRIED BREAD		AMMONIA TREATED AIR DRIED BREAD	
	ACETIC	PROPIONIC	ACETIC	PROPIONIC	ACETIC	PROPIONIC
White	25, 26 → 26	77, 78 → 78	22, 21 → 22	56, 57 → 57	41, 40 → 41	78, 79 → 79
Wheat	29	88	26	69	48	96
"100% Whole Wheat"	46	105	40	71	85	95
100% Whole Wheat Fresh Milled	28, 29 → 29	126, 128 → 127	26, 27 → 27	100, 99 → 100	84, 78 → 81	128, 121 → 126

* Method described in this report: Rapid distillation followed by chromatographic separation.

This results on plain air dried bread show slight losses of acetic acid and substantial losses of propionic acid as compared to those obtained on fresh bread. This further confirms the previous findings of volatile acid losses during air drying.(3) The ammonia treatment apparently caused an

increase of acetic acid. However, the propionic acid content was close to that found in the fresh bread.

DISTILLATION

The previously published method (1, 2) requires about two hours time for preparation of the sample solution previous to distillation. Protein precipitation, filtration or centrifuging, and finally chloride precipitation and filtration are necessary. A gravimetric formic acid determination is required to correct the titrations for this acid so that other acids may be calculated by simultaneous equations. Thus far the formic acid content has been of no significance in the interpretation of bread analysis. Now that chromatographic separation of volatile acids is possible, it remained to develop a means of complete removal of acetic and propionic acids from other components. If so desired, the distillates from the standardized Hillig procedure (4) could be also used as a basis for column partition separation. Factors are necessary to correct for the partial distillation.

Friedman (5) described a direct steam distillation for the determination of alcohols and volatile acids in fish. Magnesium sulphate was used to increase the rate of separation. Munsey has outlined a similar procedure for bread (6). The Associate Referee conducted experiments to find the most efficient system of rapid steam distillation of acetic and propionic acids. Various combinations of bread, sulphuric acid, phosphotungstic acid, and magnesium sulphate were tried. It was found that increasing amounts of magnesium sulphate gave increased percentage of acid recovery in a given volume of distillate. Propionic acid could be completely distilled in about 100 ml but it was necessary to specify 200 ml. for complete distillation of acetic acid.

RAPID DISTILLATION PROCEDURE

Transfer 10 g of air dried bread or 15 g of the prepared fresh bread (2) to a 150 ml distilling flask. Add 50 ml of water and 10 ml of ca 1 *N* H_2SO_4. Mix thoroly and add 10 ml of phosphotungstic acid (20% w/v soln). Mix by swirling and add 40 g of $MgSO_4.7\ H_2O$. Swirl again to partially dissolve the salt. The mixture should now be acid to Congo Red paper. Add $H_2SO_4(1+1)$ if necessary. Connect to condenser, insert steam delivery tube, and heat to boiling. Connect to steam generator and distill 200 ml in 35–45 min. Maintain a volume of 60–80 ml in the distilling flask by means of a small burner. The steam generator described for the standardized Hillig procedure (4) has been found convenient for this purpose. Transfer the distillate to a 400 ml or 600 ml beaker, add about 10 ml of 0.01 *N* formic acid, make alkaline to phenolphthalein with ca 1 *N* NaOH, and evaporate to about 5 ml. Transfer to a glass stoppered test tube of about 25–30 ml capacity, rinsing the beaker with three 5 ml portions of water. If insoluble material adheres, add a few drops of ca 1 *N* H_2SO_4 with one rinse. Evaporate just to dryness by inserting the tube in a steam bath or in boiling water. The use of an air jet to hasten evaporation is recommended. Proceed as directed for the chromatographic separation and determination of the acids.

PARTITION CHROMATOGRAPHY

The chromatographic procedure of Ramsey and Patterson (7) has been

used to good advantage in providing additional means of identification of volatile acids C_1–C_4. Silicic acid with water as the immobile solvent is used as a basis for the column. Normal butyl alcohol-chloroform mixtures are used as mobile solvents. Bromcresol Green or "RHN_4" indicators are used to show the positions of the developed acid bands. In that the partition coefficient between chloroform-butyl alcohol/water increases with increasing moleculor weight, the acids move down. Butyric leads and then propionic, acetic and formic following in that order. With equal amounts of silicic acid prepared in the same manner, the amount of mobile solvent required to move the leading edge of each acid band to the bottom of the column is quite constant and serves as one means of identification. This is referred to as the "Threshold Volume." The rate of movement of the bands is increased by changing to a higher concentration of the normal butyl alcohol in chloroform.

In trials of this procedure for the separation of formic, acetic, and propionic acids the main difficulty was in obtaining quantitative elution of acetic acid. The leading edges of the bands were quite sharp, but the following portion graded up towards the next acid. To obtain quantitative separation it was found necessary to carry the leading edge of each band to the bottom before changing elution receivers. Acetic acid could thus be moved down to obtain a good recovery of propionic acid. However, this could not be accomplished with formic acid to insure the complete elution of acetic acid. Increasing the percentage of normal butyl alcohol often caused the formic acid band to disappear completely. This was probably due to the small amount of formic acid (1–2 mg/10 g) normally present in bread. Later it was found that this difficulty could be over come by the addition of sufficient formic acid (about 5 mg) to give a wider band.

The general procedure for partition chromatography of acids C_1–C_4 has been adequately described (7) and needs no repetition. The following modifications have been used in the bread analyses reported here.

Mobile Solvent

Tertiary butyl alcohol 2% and 10% v/v mixtures in washed $CHCl_3$ was substituted for normal butyl alcohol. With 10% tertiary butyl alcohol

TABLE 2.—*Threshold volumes with butyl alcohol chloroform mixtures*

ACID	TERTIARY—$C_4H_{10}O$		NORMAL—$C_4H_{10}O$	
	2%	2% & 10%*	2%	2% & 10%*
	ml	*ml*	*ml*	*ml*
Propionic	18	18	19	19
Acetic	44	31	44	30
Formic	123	42	129	39

* Excess of 2% solvent poured off at propionic acid threshold and elution continued with 10% solvent.

in washed chloroform it was possible to maintain small formic acid bands intact while moving to the bottom of the column. However, comparing 2% concentrations in washed chloroform there were no appreciable differences in "Threshold Volumes" in separating a mixture of ten ml of each 0.01*N* formic, acetic, and propionic acids.

Column Material-Preparation

The amount of ammonia necessary to neutralize the silicic acid-indicator mixture is best determined by a previous titration. About 10 gm of silicic acid and 1 ml of indicator in 20 ml of water are titrated with about 0.5 *N* NH_4OH. This is preferred to estimating the change in the comparatively dry silicic acid while grinding in mortar.

It has been found desirable to prepare the column mixture of silicic acid, water, ammonia, and indicator in a batch that provides material for about twelve columns. One hundred gm silicic acid, 24 ml water, 12 ml. indicator, and 1.2 ml. 0.5 *N* NH_4OH were thoroughly mixed by grinding to an apparent uniform powder in a 6-inch mortar. The uniformity was then tested by sieving through a 40-mesh sieve. Any lumps were rubbed through. The total was then transferred to a jar with tight fitting cap and mixed by rolling in the jar. Ten gram portions of this mixture were sufficient for separation of the acids in a 12-inch tube of 14 mm internal diameter. Batch preparation of the column mixture should make for more uniform threshold volume determinations.

Elution

In that water is added to the butyl alcohol-chloroform eluates at the time of titration it was thought that this amount of water might well serve a dual purpose, *i.e.*, to provide a medium for the indicator change and to guard against acid loss by collecting the volatile solvent under water. Therefore 20 ml of water was added to each eluate receiver previous to collecting each band.

Titration

The necessity for shaking the water-butyl alcohol-chloroform mixture during the titration and frequent opening and closing of the flask as the end point was neared, resulted in CO_2 error with 0.01 *N* NaOH and phenolphthalein indicator. This can be prevented by bubbling a stream of CO_2-free air through the mixture. However, it was found that ordinary titration with phenol red (0.1%) gave comparable results.

Isolation of Volatile Acids

To the dry residue in the glass stoppered test tube, add one drop (0.05 ml) of water and about six glass beads. Stopper and swirl until all of the solid material appears moist. Add 4 ml of the butyl alcohol-chloroform solvent (2% of either normal or tertiary butyl alcohol in washed chloroform) and 3 drops of (1+1 H_2SO_4). Stopper and swirl to mix the contents without undue wetting of the top of the tube or stopper. Add about 1 gm of anhydrous Na_2SO_4 and again mix thoroly. Transfer the

solvent to the prepared column with an eye dropper pipette. Repeat the extraction with three 2-ml portions of solvent applying pressure to force each portion into the gel.

When the threshold of propionic acid has been reached, pour off the excess 2% solvent and continue the elution with 10%. If propionic acid is absent continue with the 2% solvent to the acetic acid threshold before making the change.

RECOVERY EXPERIMENTS

(Rapid Distillation and Chromatographic Separation)

The rapid distillation as above described gave 99–100% recovery of acetic and propionic acids previous to the chromatographic separation procedure.

The following recoveries were made on the basis of acetic and propionic acids added to 10 g of air dried white and wheat breads. These bread samples were prepared for the investigation previously reported (3). Ten ml of approximately 0.01 *N* formic acid was added as an aid in the column separation of acetic acid.

TABLE 3.—*Recovery of acetic and propionic acids by rapid distillation and chromatographic procedure*

BREAD	ACETIC ACID			PROPIONIC ACID		
	ADDED	RECOVERED		ADDED	RECOVERED	
	mg	*mg*	*per cent*	*mg*	*mg*	*per cent*
White Control	None	5.0	—	None	None	—
Wheat Control	None	4.9	—	None	None	—
White +Acids	13.3	17.9	97	15.1	14.0	93
White +Acids	13.3	17.2	92	15.1	14.9	99
Wheat+Acids	13.3	17.3	93	15.1	14.7	97
Wheat+Acids	13.3	—	—	15.1	14.0	93

TABLE 4.—*Acetic and propionic acids in authentic breads**

(Methods Comparison)
(mg/100 g—air dry basis)

	METHOD I		METHOD II	
	ACETIC	PROPIONIC	ACETIC	PROPIONIC
White (control)	51	None	48, 48 → 49	None
White with Sodium Propionate	53	70	45, 50 → 48	80, 79 → 80
White with Sodium Diacetate	133	None	131, 137 → 134	None

* Basis, authentic bread prepared for 1948 investigation (3).

METHODS COMPARISON

Analyses in comparison of the standardized Hillig distillation (4) followed by chromatographic separation (Method I) and the present reported rapid distillation and subsequent chromatographic separation (Method II) are compiled in Table 4.

SUMMARY

The present report covers preliminary trials of a method for determining acetic and propionic acids in bread that may be considered as alternative to the previously adopted method (1, 2). Rapid distillation to give complete recovery of the two acids is followed by partition chromatographic separation and quantitative determination. Results are reported on fresh bread as compared to the same bread air dried and on the same bread treated with ammonia vapor and then air dried. The results on air dried bread further confirm previously reported findings as to losses of volatile acids occurring during the air drying of bread. The ammonia treatment served to prevent loss of propionic acid, but appeared to increase the amount of acetic acid.

RECOMMENDATIONS*

It is recommended—

(1) That this study be continued for the following described purposes:

(a) To develop methods of preserving bread samples to insure against spoilage or the loss of volatile acids.

(b) To submit to collaborative trial the procedure outlined in this report: the determination of acetic and propionic acids in bread by rapid distillation and chromatographic separation.

(2) That the Committee on Revision of Methods be advised of the following described change deemed advisable in the method "Volatile Acids in Bakery Products" published in *This Journal*, **31**, 99 (1948). Factors for calculation given in the last paragraph apply only where a 25 g sample is used. Before publication in the 7th Ed. of *Methods of Analysis* the last paragraph should be changed to read as follows:

"Determine formic acid in a composite of the distillates and correct the titrations for the titer contributed by this acid. Calculate results in terms of mg of each acid per 100 g of sample (1 ml of 0.01 *N* is equivalent to 0.46 mg of formic acid; 0.60 mg of acetic acid, or 0.74 mg of propionic acid)."

Attention is also called to the reference in the 4th paragraph to section **24.11**, 6th Ed. Methods A.O.A.C. (A different number may be used in subsequent editions.)

REFERENCES

(1) McRoberts, L. H., *This Journal*, **31**, 489 (1948).
(2) ———, *Ibid.*, **31**, 99 (1948).

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 56 (1950).

(3) ———, *Ibid.*, **32**, 496 (1949).
(4) *Methods of Analysis, A.O.A.C.*, 6th ed., Sec. **24.10–24.11.**
(5) FRIEDEMANN, T. E., *J. Bio. Chem.*, **123**, 164 (1938).
(6) MUNSEY, V. E., U. S. Food and Drug Administration, Washington, D. C., Personal communication.
(7) PATTERSON, W. I., and RAMSEY, L. L., *This Journal*, **28**, 644 (1945).

REPORT ON THIOUREA IN FOODS

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

A rapid oxidation method for thiourea in citrus juice was reported last year and published in the February, 1949, issue of the *Journal*.[1] The method is rapid and direct and very promising. This year two samples of orange juice, containing known quantities of added thiourea, were prepared for collaborative study by the method. To the first sample 400 p.p.m. of monochloroacetic acid were added as a preservative. Carbon tetrachloride was added to the second sample. After a trial storage period the second sample containing CCl_4 was found to have more of the appearance of the fresh juice and consequently only this sample was sent out for collaborative study. The collaborators were instructed to analyze the sample by the published method, but they were asked to make two shake-out extractions with ether in place of the one called for in the method. This additional extraction was found necessary to remove traces of the preservative (CCl_4) which partially inhibited the reaction of the reagent. Collaborators were asked to carry along with the determination a sample to which they had added a known amount of thiourea. The sample contained 32 p.p.m. (32 mg/l) of added thiourea.

A few days after its preparation, the Associate Referee made the determination and found 31.25 p.p.m. of thiourea, a recovery of 97 per cent. Results were obtained from collaborators after a period of several months, which showed recoveries on the official sample of about 20 p.p.m. However, they were able to obtain good recoveries on the trial sample to which they had added a known quantity of thiourea. The collaborative sample had been stored without refrigeration in some cases.

Suspecting that deterioration had occurred, the writer reexamined a remaining portion of the sample which he had kept under refrigeration and found only 25 p.p.m. of thiourea.

These results indicate that there is a slow reaction either with constituents in the sample or with the carbon tetrachloride. The Associate Referee therefore prepared a fresh sample of juice containing the same amount (32 mg/l) of added thiourea and submitted it to collaborators for analysis. The results are shown in Table 1.

[1] *Changes in Methods of Analysis*, p. 100.

TABLE 1.—*Rccoveries of thiourea in orange juice by collaborators, using the rapid oxidation method*

COLLABORATOR	ADDED THIOUREA	THIOUREA FOUND	PER CENT RECOVERY
	p.p.m.	*p.p.m.*	
C. G. Hatmaker	32	30.8	96.2
W. O. Winkler	32	31.1	97

The results obtained by the collaborators show that good recoveries of thiourea can be obtained on samples of fresh sound orange juice. The method is therefore satisfactory for such samples. Details of the Method are published in *Methods of Analysis*, 7th. Ed. (1950).

RECOMMENDATIONS*

It is recommended—

(1) That the rapid oxidation method for thiourea in orange juice, published in *This Journal*, **32**, 100 (1949) be adopted as first action.

(2) That a further study be made to determine the cause of the lower recoveries on the preserved sample.

REPORT ON FORMALDEHYDE

By HOWARD P. BENNETT (U. S. Food and Drug Administration, Federal Security Agency, New Orleans, La.), *Associate Referee*

In accordance with recommendations of the Association, collaborative work was undertaken this year on methods to detect formaldehyde in food products. Four milk samples were prepared for this work and frozen. They were kept in a frozen condition until analyzed, when they were thawed and mixed. The analysis was carried out before the samples were two weeks old. Six different laboratories engaged in the work, eight chemists reported. The methods employed and the results on the four milk samples follow:

PREPARATION OF SAMPLE

If sample is solid or semi-solid, macerate 100 gm with 100 ml of water in mortar. Transfer to an 800 ml Kjeldahl flask, acidify with phosphoric acid, add 1 ml excess, connect with a condenser through a trap, and slowly distill 50 ml. In the case of milk, dilute 100 ml with 100 ml of water, acidify and distill as described above. With other liquids, make 200 ml acid and distill as above described.

TEST A (HEHNER-FULTON TEST)

REAGENTS

Conc. Sulfuric Acid.

Aldehyde-free milk.

Oxidizing soln: To cold conc. sulfuric acid, add in small amounts an equal volume of saturated bromine water, cooling throughout the operation.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 56 (1950).

THE TEST

To 6 ml of cold conc. sulfuric acid, add slowly and with cooling, 5 ml of the prepared distillate. Place 5 ml of this soln in a test tube, and add slowly with cooling 1 ml of aldehyde free milk, followed by 0.5 ml of the oxidizing soln. Mix. A purplish pink color indicates formaldehyde.

TEST B (TANNIC ACID TEST)

REAGENT

Dissolve 35 mg of tannic acid in 100 ml of conc. sulfuric acid.

THE TEST

To 5 ml of the reagent in a small casserole, add 1 ml of the prepared distillate. Heat on a steam bath for 5 min., or less if the test shows positive before then. The appearance of a green or blue-green color indicates formaldehyde. Make a blank determination for comparison, using 1 ml of distilled water instead of the distillate.

TEST C (CHROMOTROPIC ACID TEST)

REAGENT

Prepare a saturated soln of purified chromotropic acid (ca 500 mg/100 ml) in ca 72% sulfuric acid. A light straw-colored soln should result.

THE TEST

Place 5 ml of the reagent in a test tube and add with mixing 1 ml of the prepared distillate. Place in a boiling water bath for 15 min. Observe during the heating. The presence of formaldehyde is indicated by the appearance of a light to deep purple color, depending upon the amount present.

TEST D, A.O.A.C. 32.26 (PHLOROGLUCINOL TEST)

Results on 100 ml samples taken for analysis:

Sample No. 1 contained 10 mg of formaldehyde per kilogram. All chemists reported the four tests positive for the compound.

Sample No. 2 contained no formaldehyde and each chemist reported all tests as negative.

Sample No. 3 contained 5 mg of formaldehyde per kilogram. All chemists reported positive tests by methods A, B, and C. Two chemists reported negative and one reported doubtful by test D; all others reported positive.

Sample No. 4 contained 1 mg of formaldehyde per kilogram of milk, the results follow: (+ indicates positive; − indicates negative).

Chemist	*Test A*	*Test B*	*Test C*	*Test D*
1	doubtful	+	−	−
2	−	+	−	−
3	+	+	+	−
4	+	−	−	−
5	+	+	+	−
6	+	doubtful	+	−
7	+	−	+	+
8	+	doubtful	+	doubtful

COMMENTS OF COLLABORATORS

Cox: "Tests A and C gave definite results, and the color intensities appear to give a quantitative indication of the relative amounts of formaldehyde present . . . . Test D appears to be least satisfactory of the four tests . . . ."

Dow: "Test A seemed to be by far the most satisfactory to use. Test B was unsatisfactory because the colors obtained were difficult to interpret. Test C was satisfactory but did not seem to be as sensitive as the Hehner-Fulton test. Test D was somewhat difficult to interpret . . . ."

Bond & Yarnall: "It is the opinion of both chemists that Test B is the most sensitive, although the instability of the reagent raises a question . . . . Deterioration of the reagent was noted within an hour after preparation . . . . Test A and C are of equal value, but since the reagents employed in Test C are strictly of laboratory status, the choice as second best of the four tests lies in Test C."

Hanson: "Test C is the quickest and most definite test. Test A seems to be very delicate but it is more time consuming. Test B seems to work better with larger amounts of formaldehyde. The reagent darkens somewhat on heating on the steam bath . . . . Test D does not seem as delicate as the other tests."

Collins: Preferred Test C to the others, with A outranking B and D.

Ensminger: "Test A—The test is simple and easily performed. The tannic acid (B) test had a rather indefinite bluish-green coloration for the positive formaldehyde indication. . . . Test C . . . . Although this test consumed more time than the other three, the coloration proved quite sensitive, although not quite as sensitive as Test A. The phloroglucinal Test (D) in my estimation proved the least convincing as to indication of presence of formaldehyde . . . ."

From the foregoing one arrives at the conclusion that Test D (Phloroglucinal Test) is not very sensitive and is sometimes hard to interpret. Test B (Tannic Acid Test) is sensitive, but the reagent is unstable. Tests A (Hehner-Fulton) and C (Chromotropic Acid Test) seem to be the best from all points of view. The latter test is highly specific, a point to recommend it strongly.

It is therefore recommended* that the present methods for detection of formaldehyde in foods in the current *Book of Methods* be deleted and that the Hehner-Fulton test and the chromotropic acid test be substituted, and adopted as first action. It is also recommended that the paragraph on preparation of sample be replaced by the method outlined above and be adopted as first action. It is recommended that this subject be closed.

The Associate Referee wishes to express his thanks to H. R. Bond, F. E. Yarnall, A. W. Hanson, M. L. Dow, W. S. Cox, F. H. Collins, and L. G. Ensminger, all of the Food and Drug Administration, who collaborated in this work.

* For report of Subcommittee C and action of the Association, see *This Journal* **33** 56 (1950).

REPORT ON ARTIFICIAL SWEETENERS

By WILLIAM S. COX (Federal Security Agency, U. S. Food and Drug Administration, Atlanta, Ga.), *Associate Referee*

The status of the field of artificial sweeteners for foods has changed but little since the report by the Referee, *This Journal*, **30**, 490 (1947).

The sweetener, 1-propoxy 2-amino 4-nitrobenzene, now over two years old, is considered a toxic substance. As reported previously by the Referee, *This Journal*, **31**, 473 (1948), a method for the determination of this sweetener has been published. A more detailed scheme of the same method was published in *The Analyst*, **72**, 548–549 (1947).

No method for the detection of this sweetener has been published up to the writing of this report. The Associate Referee has made attempts at developing a method for the detection, but without appreciable success.

SACCHARIN

The recommendation of Subcommittee C, *This Journal*, **32**, 57 (1949), was followed up by a collaborative study of the method for saccharin described by the Associate Referee, *Ibid.*, **30**, 494 (1947).

For this collaborative study, a cola drink was admixed with a solution of saccharin and sodium benzoate, so that the resulting product was a noncarbonated soft drink with 40.0 milligrams of saccharin per 200 ml (amount required for analysis). Duplicate portions were sent to collaborators with instructions to determine saccharin by the method shown below, which is identical with the method recommended in the reference cited in the previous paragraph. The details of the method will be published in the 7th Ed., *Methods of Analysis*, 1950.

COMMENT OF COLLABORATORS

Analyst A: No comments.

Analyst B: There is a marked tendency for the saccharin to creep over the edge of the beaker as the alcohol evaporates. This occurs with or without the use of an air stream to hasten evaporation. The residues after drying are brown in color.

After the first 1 hour sublimation period, an appreciable quantity of sublimate was present as a ring on the inner surface of the outer tube on a level with the bottom of the cold finger. This was washed to the bottom of the tube with ether, the ether evaporated, and the sublimation continued. Two batches of sublimate appeared to be sufficient.

Analyst C: After sublimation, and on evaporating the solvent, it was observed that the residue contained a contaminant (a light dirty brown material in spots). On drying the residues at 100°, the residues were seen to smoke. Charred material was noted in these residues, and they were taken up in alcohol, filtered, transferred back into weighing flasks and reweighed.

Analyst D: An improvised sublimator was used consisting of 2 test tubes of different sizes, air flowing into smaller one—vacuum on larger one.

Vacuum obtained averaged 6–8 mm.

If combined ether extract is washed with a few ml of H_2O, much whiter crystals are obtained at that stage. Final crystals are brownish in color.

Analyst E: It is believed that the brown color present in the residues is due to the flavoring material present in the cola drink. It is doubtful that the ordinary non-cola soft drink would contain this type of material which would contaminate the saccharin residues.

The tendency of saccharin in alcohol or ether to creep over the edge of its container may be eliminated by the use of a container with a ground edge, such as a weighing flask.

The results of Analyst D are appreciably lower than any of the others reported. The agreement between the results of the other analysts is good, considering the nature of the sample. It would appear that Analyst D's inability to attain a vacuum of 1–2 mm required by the method would account for these low results.

TABLE OF RESULTS OF COLLABORATORS

Saccharin present (mg/200 ml sample): 40.0

			Per cent recovery (Average of 2)
Found by			
Analyst A	37.5		94.4
	38.0		
Analyst B	37.3		99.3
	42.1		
Analyst C	36.8		90.1
	35.3		
Analyst D	22.5	20.5	*
	29.4	23.6	
Analyst E	37.7		92.8
	36.5		

* Not averaged, since method not followed as to required vacuum.

The results, although not excellent, are probably good considering the nature of the samples examined.

The Associate Referee wishes to express appreciation to the following collaborators for their cooperation.

Douglas D. Price, Baltimore District, U. S. Food and Drug Admin.
Louis C. Weiss, Los Angeles District, U. S. Food and Drug Admin.
David Firestone, New York District, U. S. Food and Drug Admin.
J. Phyllis Skyrme, Boston District, U. S. Food and Drug Admin.

DULCIN

The A.O.A.C. *Book of Methods*, 6th Ed., does not include a method for the determination of dulcin; and a search of the literature was made, but no satisfactory method for the determination of dulcin present in less than 1% concentration was found. The Associate Referee has attempted to develop a suitable method, but without success to this date.

The *Book of Methods* includes two methods for the detection of dulcin, but experiences of the Associate Referee and other analysts of the Food

and Drug Administration have shown the first (Morpurgo) method to be almost uniformly unsatisfactory, and the second (Jorissen) method is not dependable.

A search of the literature indicates that approximately 10 methods for the detection of dulcin have been published. Of these, two have been selected and tested by the Associate Referee. These tests are simple and specific, and require reagents which are readily available. Unfortunately, lack of time allowed only limited collaborative work on these tests. However, the Associate Referee feels compelled to recommend the adoption of these tests to replace those now found in the A.O.A.C. *Book of Methods*, which have been shown to be unsatisfactory.

Details of the two methods will be published in *Methods of Analysis*, 7th Ed., 1950.

RECOMMENDATIONS*

It is recommended—

(1) That the sublimation method for saccharin described in this report be adopted, first action, for beverages, and that further study be made of the method in order to make it applicable to other foods, particularly baked goods.

(2) That methods for the determination of saccharin in baked goods be further studied.

(3) That further study be made to develop methods for the detection and determination of 1-propoxy 2-amino 4-nitrobenzene.

(4) That the methods for the detection of dulcin, **32.37,** Morpurgo Method, and **32.38,** Jorissen Method, be deleted.

(5) That the methods for the detection of dulcin included in this report (the Déniges-Tourrou Method and the Modified LaParola-Mariani Method) be adopted as first action.

(6) That methods for the determination of dulcin be further studied.

REPORT ON EGGS AND EGG PRODUCTS

By F. J. McNall (U. S. Food and Drug Administration, Federal Security Agency, Cincinnati, Ohio), *Referee*

Associate Referee Lloyd C. Mitchell made a collaborative study of the method for fat by acid hydrolysis as applied to dried whole eggs and frozen whole eggs. A statistical study was also made of the collaborative results which indicates that the method is capable of giving excellent recoveries in the hands of different analysts. The Associate Referee recommends that the method be made official and the Referee concurs.

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* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 55 (1950).

ADDED GLYCEROL IN EGGS

Associate Referee George E. Keppel made a collaborative study of the present tentative qualitative test for glycerol in eggs, and of the proposed quantitative periodate method which was submitted by the Associate Referee last year. The periodate procedure has many advantages over the present tentative dichromate oxidation method. Good collaborative results were reported by these studying the periodate method and the Associate Referee recommends that the present tentative method be dropped and that the proposed method be made first action. This recommendation is approved.

The Associate Referee recommends that the present qualitative test for glycerol, **23.25** and **23.26,** be changed to include the use of basic fuchsin, that potassium bisulfate be used in place of potassium sulfate, and that the reference to crystallization of sugar be omitted. The Referee approves of these changes in the method. The Committee on Revision proposes to label the "Glycerol Qualitative Test" as a "Procedure" and the Referee approves of this change.

ACIDITY OF ETHER EXTRACT

Method I Official **23.29** and **23.30,** and Method II Rapid Method, Tentative **23.31** and **23.32** were studied collaboratively by Associate Referee Halver C. Van Dame with a view of formulating a consolidated procedure, retaining the best features of each method. The results submitted by the Associate Referee indicate that the Rapid Method for dried eggs **23.32**(a) is just as satisfactory as the Official Method **23.30**(a) and has the advantage of being much more rapid. However, in the case of liquid eggs the Associate Referee proposes that the eggs be dried at 55°C. at a pressure not exceeding 125 mm of mercury for approximately five hours and then proceed as in Method II, **23.32**(a).

The Associate Referee recommends that Method II, **23.32(a),** for dried eggs, as modified in his report, be made official for dried eggs, that the method for liquid eggs as given in his report be made official for liquid eggs, and that **23.30, 23.31** and **23.32(b)** be deleted and the subject closed. The Referee concurs in all these recommendations.

AMMONIA NITROGEN

Associate Referee E. B. Boyce has submitted a proposed method which is a modification of the original Folin method, and has been used successfully in the Massachusetts Department of Public Health laboratories as an index of protein decomposition in fluid eggs. The Associate Referee recommends that the present tentative method **23.33** and **23.34** be dropped, and that the proposed method be studied collaboratively next year. The Referee concurs in this recommendation.

The Referee on Decomposition of Fish Products is recommending the

deletion of the determination of volatile acids by the empirical distillation method which employs the calculation of the acids by simultaneous equation. This procedure is part of the method for the determination of volatile acids in eggs (**23.37**). Also, the Referee on Decomposition is recommending the adoption of a chromatographic method for the separation and determination of these acids. Further, the distillation procedure can be somewhat simplified when the distillate is subjected to chromatography. Accordingly the present method **23.36–23.37** should be deleted and the method, recommended by the Associate Referee on decomposition of eggs for volatile acids adopted.

RECOMMENDATIONS*

It is recommended—

(1) That "Collection and Preparation of Sample—Tentative" **23.1** be changed to "Collection and Preparation of Sample—Procedure."

(2) That "Water-Soluble Nitrogen and Crude Albumin Nitrogen—Tentative for Dried Eggs" **23.6(b)** be dropped.

(3) That "Fat by Acid Hydrolysis—Tentative" **23.8** and **23.9** be made official.

(4) That the present tentative method for lipoids and lipoid phosphoric acid, **23.10, 23.11,** and **23.12,** be made first action.

(5) That "Glycerol Qualitative Test—Tentative" **23.25–23.26** be changed to "Glycerol Qualitative Test—Procedure" and that the slight modifications suggested by the Associate Referee be made.

(6) That the present tentative quantitative method for glycerol, **23.27, 23.28,** be dropped and the proposed periodate method be made first action.

(7) That the present methods for acidity of ether extract **23.30, 23.31** and **23.32(b)** be dropped and that **23.32(a)** as modified by the Associate Referee, together with his proposed method for liquid eggs, be made official, and that the subject be closed.

(8) That the present tentative method for ammonia nitrogen, **23.33** and **23.34,** be dropped and the proposed method of the Associate Referee be studied collaboratively next year.

(9) That the method for volatile fatty acids **23.36** and **23.37** be dropped and the method described by the Associate Referee on decomposition of eggs be adopted, first action.

(10) That the tentative method for extraction and identificatior of added color, **23.38,** be dropped. This is a cross reference to the cereal chapter and has never been studied collaboratively as applied to eggs.

(11) That further work be done on methods for glycerol in egg mixtures containing added sugars.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 49 (1950).

REPORT ON ADDED GLYCEROL IN EGGS

By GEORGE E. KEPPEL (U. S. Food and Drug Administration, Federal Security Agency, Minneapolis 1, Minnesota), *Associate Referee*

The previous report of the Associate Referee[1] outlined a tentative method for isolating and determining glycerol added to whole egg or egg yolk. Satisfactory recoveries were obtained on a series of prepared samples. Committee C recommended that the method be tested collaboratively.

It was also recommended that the tentative qualitative method for glycerol[2] be tested similarly.

To complete the preliminary work on the method a comparison of recoveries by the present tentative dichromate method[3] and the proposed periodate method, was made. The results obtained are given in Table 1.

TABLE 1.—*Results on glycerol recoveries by tentative $K_2Cr_2O_7$ method and by proposed KIO_4 method*

PRODUCT	ADDED GLYCEROL	GLYCEROL FOUND				RECOVERY, %			
		$K_2Cr_2O_7$ METHOD		KIO_4 METHOD		$K_2Cr_2O_7$ METHOD		KIO_4 METHOD	
		1	2	1	2	1	2	1	2
Whole Egg	0	0.37		0.17					
Yolk	0	0.30		0.10					
Whole Egg and Glycerin	9.50	9.15	9.17	9.48	9.45	97.0	97.2	99.8	99.5
Yolk and Glycerin	4.11	4.13	4.26	4.16	4.16	100.5	103.6	101.2	101.2
Same	8.85	8.60	8.65	8.86	8.86	97.2	97.7	100.1	100.1
Same	14.23	13.76	14.09	14.11	14.19	96.7	99.0	99.2	99.7
Average:						98.6		100.1	

The samples were prepared from fresh eggs, broken and separated in the laboratory. The added glycerin (U.S.P.) had a strength of 95.0% by periodate oxidation, 94.9% by specific gravity measurement, and 95.1% by dichromate oxidation.

The results indicate that recovery is slightly less by the dichromate method over a range of glycerol concentration likely to be encountered in commercial products. The dichromate method, being less specific, requires a rather complicated isolation technique. A potential source of error is the high coefficient of expansion of the strong dichromate solution unless all volumetric measurements are made at the same temperature.

[1] *This Journal*, **32**, 506 (1949).
[2] *Methods of Analysis A.O.A.C.* (1945), 23.25 23.26
[3] *Ibid.*, 23.27, 23.28.

COLLABORATIVE STUDY

A series of samples for collaborators was prepared by thoroughly mixing weighed quantities of fresh egg yolk or whole egg with glycerin, U.S.P., of known strength. The mixing was accomplished in closed containers by shaking. After standing for two days in a refrigerator, portions were transferred to friction top metal cans and frozen in a sharp freezer. The samples were shipped in a frozen condition, packed with dry ice. Sample 1 consisted of whole egg with 9.0% added glycerol; sample 2 was egg yolk with 5.0% glycerol; sample 3 consisted of whole egg only. Each collaborator was asked to make qualitative tests for glycerol on all three samples using the tentative A.O.A.C. Method **23.25** and **23.26.** The samples found to contain glycerol were to be examined by the proposed method, which is essentially the same as that previously submitted by the author, except that cross reference is made to Bruening's method[4] for the actual determination of the isolated glycerol. The test for excess periodate is essential when the approximate amount of glycerol present is not known.

The results obtained by the collaborators for the qualitative tests are shown in Table 2, and quantitative results are given in Table 3.

TABLE 2.—*Collaborative results on qualitative test for glycerol*

COLLABORATOR	SUB NO. 1	SUB NO. 2	SUB NO. 3
1	Positive	Positive	Negative
2	Positive	Positive	Negative
3	Positive	Positive	Negative
4	Positive	Positive	Positive

TABLE 3.—*Collaborative results on quantitative method for glycerol*

COLLABORATOR	SAMPLE 1		SAMPLE 2	
	GLYCEROL FOUND	RECOVERY	GLYCEROL FOUND	RECOVERY
	per cent	*per cent*	*per cent*	*per cent*
1	9.07	100.8	5.22	104.4
			5.16	103.2
2	8.62	95.8	4.90	98.0
	8.71	96.8	4.93	98.6
3	8.86	98.4	4.95	99.0
	8.99	99.9	5.01	100.2
4	8.78	97.6	4.98	99.6
	8.91	99.0	5.08	101.6
5	9.08	100.9	5.11	102.2
	8.95	99.4	5.18	103.6
	9.01	100.1		
6	9.04	100.4	5.13	102.6
	9.01	100.1	5.15	103.0
Ave.	8.92	99.1	5.07	101.4

[4] *This Journal*, 31, 72 (1948).

COLLABORATORS

(1) H. I. Macomber, Baltimore, Md.
(2) F. J. McNall, Cincinnati, Ohio.
(3) L. C. Mitchell, Minneapolis, Minn.
(4) F. E. Yarnell, Kansas City, Mo.
(5) W. Horwitz, Minneapolis, Minn.
(6) G. E. Keppel, Minneapolis, Minn.
(All collaborators are members of U. S. Food and Drug Administration.)

DISCUSSION OF RESULTS

Three of the four collaborators reporting on the qualitative test obtained correct results on the samples. Two of the collaborators commented as follows:

F. E. Yarnell.—"A.O.A.C. **23.25** does not indicate whether acid or basic fuchsin is used for the reagent. The U.S.P. reagent of the same name (p. 838) specifies basic fuchsin, which the U.S.P. (p. 767) describes as '. . . a mixture of rosanilin and pararosanilin hydrochloride.'

"For the qualitative tests on these samples, Eastman's rosaniline hydrochloride (practical) was used. Two solutions, one 20 hours old and one 1 hour old, were used on sample 3, and in each instance a definite darkening of the pink color occurred, when compared to the same volume of reagent in an equal volume of water."

L. C. Mitchell.—"It is noted that the method uses '15 ml of mixture of equal volumes of absolute alcohol and chloroform—to permit crystallization of sugar.' Apparently what crystallizes out is potassium chloride, a normal ingredient of eggs, and not sugar. At least when 20 ml of a solution containing 0.5 ml glycerol and 40 mg sucrose were evaporated down and treated with 15 ml of equal parts of absolute alcohol and chloroform, the resulting mixture remained clear. However, when 40 mg potassium chloride was added to a similar solution and evaporated down and treated with 15 ml of the alcohol-chloroform mixture, it was precipitated, but not the sucrose. It was also noted that when the residue in the test tube was heated with K_2SO_4, it did not froth nor did the contents become liquid."

The Associate Referee experienced no difficulty in applying the qualitative method to the three egg samples, two of which contained glycerol. The method is unique in one respect, in that potassium sulfate, instead of potassium bisulfate, is used as a dehydrating agent to produce acrolein for the test. Both reagents work equally well, but in view of the fact that U.S.P. and other present-day tests for glycerol specify potassium bisulfate, it would be advisable to use this reagent instead. For the same reason, basic fuchsin should be specified to conform to U.S.P. fuchsin-sulfurous acid test solution, which is prepared in exactly the same manner. The material used by the author was Fuchsin Merck, medicinal. This is the "basic" fuchsin, and it was found to give satisfactory results on various known mixtures.

The results on the quantitative method indicate satisfactory recoveries of glycerol from whole egg or yolk containing no added sugar. One collaborator reported that his 0.02 *M* KIO_4 solution had a distinctly alkaline reaction. A 25-ml aliquot in presence of propylene glycol required 0.7 ml of 0.2 *N* H_2SO_4 to bring to neutral point with bromcresol purple indicator.

A periodate solution prepared from a different lot of KIO_4 gave a neutral reaction. No other collaborators reported this difficulty, but a test for alkalinity in the 0.02 *M* KIO_4 has been included.

It would be desirable to devise a method applicable in the presence of added sugars. Lesser[5] describes mixtures of egg yolk with glycerin and corn sugar, as well as mixtures with glyceryl monostearate or distearate. Some experiments have been made by the Associate Referee on the removal of sucrose from egg, sucrose and glycerin mixtures, but no satisfactory method has been developed as yet.

RECOMMENDATIONS*

It is recommended—

(1) That the present qualitative test **(23.25, 23.26)** be adopted as first action, after making the following changes:

23.25. Change first sentence beginning:"—Dissolve 0.2 g of fuchsin—" to read: "—Dissolve 0.2 g of basic fuchsin—."

23.26. 10th line. Omit "to permit crystallization of sugar."

13th line. Change "powdered K_2SO_4 " to "powdered $KHSO_4$."

(2) That the proposed quantitative method for added glycerol be adopted as first action.

(3) That the present tentative method **(23.27, 23.28)** be deleted.

(4) That further work be done on methods for glycerol in egg mixtures containing added sugars.

ACKNOWLEDGMENT

The author wishes to express his appreciation to the collaborators who generously assisted in this work.

REPORT ON THE ACIDITY OF ETHER EXTRACT IN EGGS

By HALVER C. VAN DAME (U. S. Food and Drug Administration, Federal Security Agency, Cincinnati, Ohio), *Associate Referee*

Subcommittee C made the recommendation that paragraphs **23.29** and **23.30** (Acidity of Ether Extract, Method I. Official) and **23.31** and **23.32** (Acidity of Ether Extract Method II, Tentative, Rapid Method) be studied with a view toward formulation of a consolidated procedure retaining the best features of each method. This report gives the results of that study.

The Associate Referee analyzed five samples of dried eggs and three samples of liquid eggs by Methods I and II **(23.30, 23.32)** and modifications and combinations of these methods. The following table gives the results of these analyses.

[5] "The Use of Glycerinated Eggs," Lesser, Milton. *American Egg and Poultry Review*, October, 1948.

* For report of Subcommittee C, and action of the Association, see *This Journal*, 33, 49 (1950).

TABLE 1.—*Collaborative results*

SAMPLE NO.	METHOD A		METHOD B		METHOD C		METHOD D	
	% ETHER EXTRACT	ML 0.02 *N* Na ETHYLATE PER G EXT.	% ETHER EXTRACT	ML 0.02 *N* Na ETHYLATE PER G EXT.	% ETHER EXTRACT	ML 0.02 *N* Na ETHYLATE PER G EXT.	% ETHER EXTRACT	ML 0.02 *N* Na ETHYLATE PER G EXT.
Egg Yolks:								
1	50.92	6.88	51.16	6.45		6.50		6.32
	51.38	6.86	51.35	6.47				
2	50.87	2.68	50.17	2.23		2.29		2.21
	50.84	2.75	49.79	2.23				
Dried whole eggs:								
3	35.25	2.97	35.34	2.44		2.43		2.32
	35.58	2.87	35.93	2.47				
4	34.74	1.48	35.06	1.23		1.30		1.11
	34.59	1.49	34.84	1.22				
5	37.57	23.32	36.93	24.13				
	36.87	23.50	36.87	23.97				
	METHOD E		METHOD F		METHOD G		METHOD H	
Liquid eggs:								
6	10.00	1.78	9.83	1.25	9.37	1.35	12.52	1.48
	10.19	1.75	9.86	1.25	9.45	1.50	13.02	1.38
7	9.40	1.50	10.37	1.42	7.70	1.23	8.49	1.22
	9.80	1.43	10.20	1.50	7.15	1.21	8.42	1.28
8	12.08	2.89	11.06	2.43				
	12.11	2.89	10.93	2.49				

Method A is Method I (**23.30(a)**).

Method B is Method II (**23.32(a)**).

Method C is Method II (**23.32(a)**) modified so that the extract is dried 1 hour at 55° as in Method I (**23.30(a)**) instead of 30 min at 100°.

Method D is Method II (**23.32(a)**) modified so that the original sample is dried as in Method I (**23.30(a)**) and extracted as in Method II (**23.32(a)**).

Method E is the method for liquid eggs described under *Methods* in this report.

Method F is Method II (**23.32(b)**) using 10 ml of alcohol to reduce emulsions.

Method G is Method II (**23.32(b)**) using no alcohol.

In studying the results of Table 1 it can be seen that although the results are slightly lower by Method B, they agree well with those of Method A. The amount of extracted material is practically the same by both

methods. The collaborative results obtained in 1932[1] also show these two methods to give comparable results.

The drying of the eggs at 55° as in Method D and the drying of the ether extract as in Method C did not seem to have any appreciable effect on the results obtained. In comparing the results on the samples of liquid eggs several conclusions were reached. Method F gave results comparable to those obtained by Method E. In Methods G and H the amount of ether extract varied considerably from that obtained by Method E. The results on the acidity of the extract were not conclusive. Since these two methods use alcohol, and petroleum ether, the extract obtained cannot be considered as an ether extract.

The Referee decided to send out 2 samples of dried eggs and 1 sample of liquid eggs to collaborators to be analyzed by the following methods:

METHODS

Dried Eggs. Method II **(23.32(a))** with the following changes: (1) change "ether 2–3 times, using ca 15 ml each time" to "3, 20 ml portions of ether." (2) change "dry flask containing extract 30 min. at 100°" to "dry extract 15 min. at 100°C."

Liquid Eggs. Weigh ca 8 g of liquid eggs into a 9 cm lipped porcelain evaporating dish. Dry at 55° under pressure not exceeding 125 mm of Hg. until eggs are thoroly dry, ca 5 hrs. Grind dried eggs in evaporating dish with small pestle. Proceed as in Method II **(23.32(a))** for dried eggs beginning "Add 30 ml of ether and mix well."

TABLE 2.—*Collaborative results*

COLLABORATOR	SAMPLE I DRIED EGGS		SAMPLE II DRIED EGGS		SAMPLE III LIQUID EGGS	
	ML 0.02 *N* Na ETHYLATE PER G ETHER EXTRACT					
1	3.34	3.29	24.36	24.46	2.32	2.44
2	3.39	3.32	24.35	24.29	2.32	2.42
3	3.21	3.19	24.12	24.13	2.68	2.60
4	3.13	3.17	23.75	23.70	1.75	1.57
5	3.24	3.36	24.30	24.33	2.16	—
6	2.53	2.55	24.55	24.57	2.17	2.09
Av.	3.14		24.24		2.23	

PREPARATION OF SAMPLES

The samples of dried eggs were thoroughly mixed by sieving 3 times and placed in small glass bottles. The sample of liquid eggs was prepared from eggs bought on the open market, broken and allowed to stand in the laboratory for several days until quite putrid. The eggs were well mixed, placed in small jars, and all frozen in the freezing unit of a refrigerator. The samples were sent to the collaborators in a frozen condition under dry ice with instructions to keep frozen until analyzed. Table 2 shows the results of the collaborators.

[1] *This Journal*, 15, 341 (1932).

CONCLUSIONS

The collaborative results on Method II **(23.32(a))** modified as given in this report, are in good agreement except for one result of collaborator 6 on Sample No. 1. The work of the Associate Referee and previous collaborative results show this method to compare very well with the present official Method I **(23.30(a))**. This method is more rapid than Method I **(23.30(a))** and can be run in hot weather when Method I **(23.30(a))** is difficult unless the laboratory is equipped with running ice water.

Four of the collaborators showed close agreement on the method proposed for liquid eggs. One collaborator got low results and one somewhat higher results. Collaborators 1 and 2 analyzed the sample on the same day only 24 hours after it had been frozen. Their results are in good agreement. The duplicate results of each collaborator are also in good agreement. Collaborators 5 and 6 analyzed their samples 2 weeks later than the others.

The Associate Referee has shown that this method is comparable to the present official Method I **(23.30(b))**. It has the same advantages that Method II **(23.32(a))** has over Method I **(23.30(a))**.

ACKNOWLEDGMENT

The Associate Referee wishes to thank L. C. Mitchell, Franklin J. McNall, Luther G. Ensminger, Ruth E. Harmon, and Hugh M. Boggs, all of the U. S. Food and Drug Administration, for their work on this subject.

RECOMMENDATIONS*

It is recommended—

(1) That Method II **(23.32(a))** with the changes made in this report be made first action for dried eggs.

(2) That the method for liquid eggs given in this report be made first action.

(3) That paragraphs **23.30, 23.31,** and **23.32 (b)** be deleted from the 7th Ed., *Methods of Analysis*, 1950.

(4) That the subject be closed.

REPORT ON FAT BY ACID HYDROLYSIS IN EGGS

By Lloyd C. Mitchell (U. S. Food and Drug Administration, Federal Security Agency, Minneapolis 1, Minnesota), *Associate Referee*

The present method **23.8** and **23.9** was made tentative in 1933[1] with a recommendation that further collaborative work be done.

This year's collaborative work was designed with a view of a statistical

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 51 (1950).

[1] *This Journal*, **16**, 514 (1933).

study of the results. Two types of eggs were submitted to the collaborators: dried whole eggs and frozen whole eggs. The contents of a one pound can of commercial dried whole eggs was passed through a twelve mesh sieve, placed in a six liter flask and thoroughly mixed by tumbling the flask for about an hour. The mixed material was dumped on paper and portions were transferred to twelve two-ounce screw cap jars until all of the original material was distributed. The frozen whole eggs were prepared in the laboratory from twelve dozen one-day-old eggs. The eggs, broken out in lots of twelve and mixed slowly with a mechanical stirrer, were poured on a forty mesh sieve to remove membranes and chalazas and the strained eggs collected in a large container where they were slowly mixed with the mechanical stirrer for about an hour. Small portions of the mixed eggs in the large container were transferred to twelve two-pound friction-top cans. The cans were placed immediately into a sharp freezer. Each set of samples was numbered alternately A and B. The cans of frozen eggs were packed with dry ice for express shipment to the collaborators; the jars of dried eggs were mailed. Two sets of dried and frozen eggs numbered A and B were sent to each participating laboratory

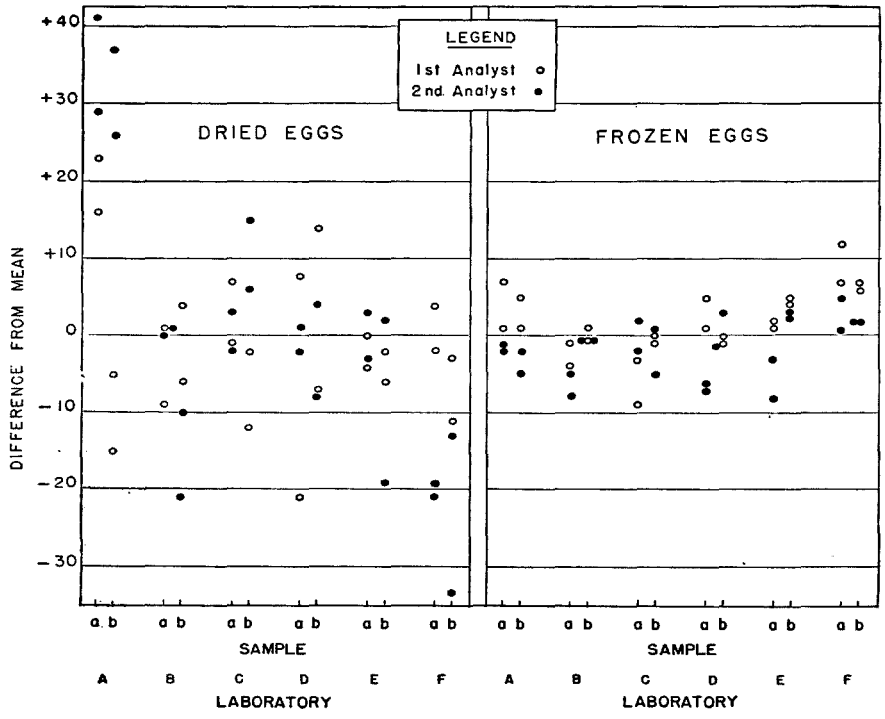

FIG. 1.—Fat by Acid Hydrolysis.

to be analyzed in duplicate by each of two collaborators in accordance with *Book of Methods*, **23.8** and **23.9.** The only instructions to the collaborators were to prepare the frozen egg samples in accordance with **23.1(b),** holding the temperature below 50° to avoid coagulation of the proteins.

RESULTS AND DISCUSSION

The results obtained from twelve collaborators in six laboratories are given in two ways: Table 1 lists all of the individual results and Figure 1 presents the data graphically as differences from the average of all of the results for the two types of eggs.

In order to evaluate the significance of (1) variations between dupli-

TABLE 1.—*Individual results on collaborative egg samples*

LABORATORY	COLLABORATOR	SAMPLE	FAT BY ACID HYDROLYSIS			
			DRIED EGGS		FROZEN EGGS	
A	1	A	42.32	42.25	11.75	11.81
		B	42.04	41.94	11.79	11.75
	2	A	42.50	42.38	11.73	11.72
		B	42.46	42.35	11.72	11.69
B	3	A	42.00	42.10	11.70	11.73
		B	42.03	42.13	11.75	11.73
	4	A	42.09	42.10	11.66	11.69
		B	41.99	41.88	11.73	11.73
C	5	A	42.16	42.08	11.71	11.65
		B	41.97	42.07	11.74	11.73
	6	A	42.07	42.12	11.76	11.72
		B	42.15	42.24	11.75	11.69
D	7	A	41.88	42.17	11.79	11.75
		B	42.23	42.02	11.73	11.74
	8	A	42.10	42.07	11.67	11.68
		B	42.01	42.13	11.77	11.73
E	9	A	42.05	42.09	11.76	11.75
		B	42.07	42.03	11.78	11.79
	10	A	42.12	42.06	11.66	11.71
		B	41.90	42.11	11.77	11.76
F	11	A	42.07	42.13	11.86	11.81
		B	41.98	42.06	11.80	11.81
	12	A	41.88	41.90	11.79	11.75
		B	41.96	41.76	11.76	11.76
Maximum			42.50		11.86	
Minimum			41.76		11.65	
Average			42.09		11.74	

cate samples run by the same collaborator (how well a single analyst can check himself), (2) variations between collaborators within a laboratory (how well two analysts in the same laboratory can check one another), and (3) variations between laboratories (how well two laboratories can check each other), analyses of variance were performed on the data for both the dried and frozen eggs. These three most important variance components, given in Table 2, are shown in three ways: in terms of the standard deviation, in terms of variations to be exceeded 1 time in 20 (*i.e.*, limits within which one would expect the check determinations

TABLE 2.—*Expected standard deviations and coefficient of variations ×100; fat by hydrolysis in eggs*

CHECK RESULTS TO BE OBTAINED	DRIED EGGS AVERAGE FAT—42.09%			FROZEN EGGS AVERAGE FAT—11.74%		
	STANDARD DEVIATION	P=.05 LIMITS	COEFFICIENT OF VARIATION×100	STANDARD DEVIATION	P=.05 LIMITS	COEFFICIENT OF VARIATION×100
By one analyst checking self	±0.10	±0.20	±0.24	±0.036	±0.07	±0.31
Between analysts within one laboratory	±0.13	±0.25	±0.31	±0.042	±0.08	±0.36
Between two laboratories	±0.15	±0.29	±0.36	±0.045	±0.09	±0.38

to fall 19 times out of 20, here called the P=.05 limits), and in terms of coefficient of variation to show the variation on a comparable basis for the two types of eggs (obtained by dividing the standard deviation by the average amount of fat and multiplying by 100).

As an explanation of the use of Table 2, the following illustration can be given. Suppose one analyst examines a sample of dried (or frozen) eggs for fat by acid hydrolysis and finds it to contain 42.09 (or 11.74) per cent fat. One would expect another analyst at the same laboratory to get a result above 42.34 (or 11.82) per cent, or below 41.84 (or 11.66) per cent, respectively, for dried and frozen eggs, only one time in 20 (42.09 ± 0.25, or 11.74 ± 0.08 per cent), respectively). The same analyst should expect to obtain a result at another time on the same sample of dried or frozen eggs between 42.29 (or 11.81) per cent, and 41.89 (or 11.67) per cent (42.09 ± 0.20, or 11.74 ± 0.07 per cent, respectively), while two analysts in different laboratories should expect to obtain a result on the same of dried or frozen eggs between 42.38 (or 11.83) per cent and 41.80 (or 11.65) per cent (42.09 ± 0.29 or 11.74 ± 0.09 per cent, respectively).

Larger limits could be taken that could be exceeded only one time in one hundred by taking a larger multiple of the standard deviation (*i.e.*, 2.58 times the standard deviation for P=.01). The P=.05 limits in Table 2 were obtained by using 1.96 times the standard deviation.

ACKNOWLEDGMENT

Grateful acknowledgment is due the following collaborators (all of the Food and Drug Administration): Earl W. Coulter and Harry O. Moraw, Chicago; Franklin J. McNall and Halver C. Van Dame, Cincinnati; Henry R. Bond and Floyd E. Yarnall, Kansas City; George E. Keppel and Sam H. Perlmutter, Minneapolis; Howard P. Bennett and George McClellan, New Orleans, and N. Aubrey Carson and Frederick M. Garfield, St. Louis. Acknowledgment is also due to Lila Knudsen, Washington, and William Horwitz, Minneapolis, for the help rendered in the statistical study of the results.

RECOMMENDATION*

It is recommended—

(1) That the tentative method **23.8** and **23.9** for fat by acid hydrolysis be made official.

REPORT ON FREE AMMONIA IN EGGS

By E. B. Boyce (State Department of Public Health, Boston 33, Mass.), *Associate Referee*

The present method for the determination of free ammonia in eggs **(23.33–23.34)** has been subject to very little collaborative study since the publication of Bulletin No. 846 of the U. S. Department of Agriculture in 1920 by Redfield. The basic method was originally developed by Otto Folin for the determination of "Free ammonia" in urine.[1] In August 1918, Hendrickson and Swan[2] published an aireation procedure for the determination of ammonia in eggs. The procedure studied and recommended by Redfield and incorporated into the present tentative method is essentially that of Hendrickson and Swan. In 1936, Joseph Calloway, Jr.[3] as Associate Referee on Detection of Decomposition in Eggs, prepared four samples of mixtures of shell eggs and distributed them to two collaborators who made independent determinations of free ammonia at approximately the same time with reasonably concordant results. Calloway called attention to the importance of complete aireation and recommended that 20 ml. of .02 *N* soln of ammonium chloride be run simultaneously and aireation be continued for a time sufficient to remove 95% of the ammonia. This "Check" assumes that ammonia would be removed from an aqueous solution at the same rate that it would be from a sample of eggs.

The modification of the Folin original method, which was developed by Hermann C. Lythgoe and which is used in the Massachusetts Depart-

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* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 51 (1950).

[1] Folin, Otto, *Z. Physiol. Chem.*, **37**, 161 (1902); *J. Biol. Chem.*, **11**, 493 (1912).

[2] Hendrickson and Swan, *Ind. Eng. Chem.*, **10**, 614 (1918).

[3] Calloway, Joseph, Jr., *This Journal*, **19**, 201 (1936).

ment of Public Health as an index of protein decomposition and of egg quality, is a somewhat simpler procedure than the present tentative method and is well adapted to the routine examination of samples. With a suitable multiple aireation unit as many as twenty samples may be examined by one chemist on the same day that the samples were obtained. Such apparatus has been in use in the Massachusetts Department of Public Health laboratories to my own knowledge for over twenty years and the details of the procedure have been requested by and given to several of the other States. The practical value of this test is self evident. The method used is as follows:

PROPOSED METHOD

APPARATUS—AIREATION

Glass cylinders approximately 300 mm high and 40 mm in diameter are fitted with two-hole rubber stoppers. Through one hole of the stopper, glass tubing extends nearly to the bottom of the cylinder. The portion of this tube extending outside of the cylinder for about four inches may be bent for convenient attachment to the multiple aireation unit. Through the other hole of the stopper is passed the end of a Kjeldahl distillation trap. To the other end of the trap is connected a piece of glass tubing which extends nearly to the bottom of a 100 ml vol. flask.

A convenient multiple aireation unit may be constructed with a section of ¾ inch gas pipe from which gas nipples are tapped at four inch intervals, each nipple being joined to the main line with a shut off valve. With an air pump of suitable capacity air is bubbled thru 35% sulphuric acid to remove any ammonia and supplied to the pipe line under pressure. A U-tube partially filled with mercury connected into the system will indicate the air pressure.

PREPARATION OF THE SAMPLE

Shell eggs are cracked into a Waring Blender or other suitable mixture and mixed for one to two minutes. A portion of frozen eggs may also be rapidly thawed and mixed in the blender.

REAGENTS

Ammonium sulphate.—The pure salt may be prepared by adding sodium carbonate to a solution of ammonium chloride and passing the liberated ammonia by aireation into a dilute soln of C.P. sulfuric acid until the acid is neutralized. The ammonium sulfate is precipitated by the addition of an equal volume of alcohol and the salt filtered and dried.

Ammonium sulfate standard soln.—Dissolve 0.1552 g of the pure salt in 500 ml of distilled water. Five ml of this soln will contain 0.4 mg of ammonia.

Hydrochloric acid.—0.1 *N.*

Nesslers soln.—Prepare as in **12.58.**

Potassium oxalate soln.—Saturated aqueous soln.

Sodium carbonate soln.—Saturated aqueous soln.

Petroleum ether.—Boiling point should be well above the temp. of the laboratory and should be checked to insure absence of any volatile substances which affect Nesslers soln.

PROCEDURE

Weigh 20 g of the well mixed sample and transfer into the aireation cylinder with a minimum of distilled water. Add 2 ml of potassium oxalate soln, 10 ml of petroleum

ether, and 5 ml of saturated sodium carbonate soln, and a small amount of an anti-foam reagent, as recommended by P. A. Racicot and C. S. Ferguson,[4] in which hexyl alcohol is incorporated into a mixture of paraffin and heavy mineral oil. (Normal butyl-phthalate or normal butyl-phosphate may be substituted for the hexyl alcohol as the active ingredient.) The rubber stopper is quickly inserted and the glass tube attached to the Kjeldahl trap is placed in a 100 ml vol. flask containing 2 ml of 0.1 *N* HCl and 60–70 ml of distilled water. The air pump is started and the inlet tube of the cylinder connected to the aireation unit with the individual valves closed to the cylinders in use, others being left open to relieve the pressure. By opening the individual valves slightly adjust the aireation at the rate of 1–2 bubbles a second, and continue slow aireation for a half hour. The rate is then increased gradually until the volume of air passing thru the apparatus is as great as possible without undue frothing or loss of the HCl soln by spattering. Continue aireation for two more hours at this vigorous rate. A reagent "Blank" is run with each run.

At the end of the aireation period the flasks are removed and the ammonia determined colorimetrically by Nesslerization. The color is matched in a visual colorimeter against a standard ammonium sulphate soln containing 0.4 mg of ammonia in 60 ml of distilled water and 2 ml of HCl. The same amount of standard soln is added to the reagent blank. To each flask is added a mixture of 5 ml of Nessler soln and 20 ml of distilled water. After Nesslerizing the flasks are made up to volume and the color comparison made.

By this method 79 samples of fresh eggs were examined. Part of these samples were examined immediately after they were obtained from a farmers' cooperative egg auction, and part were obtained in retail stores which had purchased them from this particular egg auction less than one week before. The distribution of ammonia content of these samples was as follows:

RANGE OF VALUES	NUMBER IN THE GROUP
mg ammonia per 100 gram of egg:	
1.0–1.1	6
1.1–1.2	13
1.2–1.3	19
1.3–1.4	19
1.4–1.5	12
1.5–1.6	7
1.6–1.7	2
1.7–1.8	1
Above 1.8	None

If the samples taken from retail stores are excluded from the above compilation all values fall between 1.0 and 1.5 mg per 100 grams of egg. At the time the above samples were taken additional eggs were taken at random and brought back to the laboratory where they were stored at 45°F. At the end of 4½-weeks' storage 12 eggs were picked at random and separate analysis made on each egg. The lowest value for free ammonia was 1.46 mg per 100 gram and the highest value 1.99 mg. After 2½ weeks'

[4] *Ind. Eng. Chem.*, Anal. Ed., 10, 380 (1938).

further storage (7 week total) 21 separate analyses were made on samples from the remaining eggs. At this time the lowest value was 1.68 mg and the highest 2.34 mg.

It appears that the free ammonia content of strictly fresh eggs varies within certain limits which are not too wide to be of value as a criterion of freshness, and that the free ammonia content increases in an orderly manner as the egg deteriorates. Unless the analyst has considerable first hand knowledge about the rate at which ammonia is formed, as well as knowledge concerning the temperature at which any particular sample had been stored, the age of the sample cannot be computed. The amount of ammonia does however, serve as an index of the decomposition of the yolk protein. Because the free ammonia is driven out of the egg during the drying process, the determination is of little or no value when applied to dried eggs.

In order to test the reproducibility of the method in the hands of one chemist two samples of shell eggs from the same source, each consisting of nine eggs, were mixed separatedly in a Waring blender and designated as samples A and B. Five 20 gram portions were removed from each sample and the ammonia content determined with the following results:

SAMPLE A	DETERMINATION NO.	MG NH_3 PER 100 G	DEVIATION FROM MEAN
	1	1.52	.03
	2	1.57	.02
	3	1.56	.01
	4	1.55	.00
	5	1.54	.01
Average		1.55	
	1	1.53	.01
	2	1.53	.01
	3	1.50	.02
	4	1.47	.05
	5	1.56	.04
Average		1.52	

SUMMARY

A modification of the original Folin procedure for the determination of free ammonia in urine is adapted to the determination of free ammonia in liquid eggs. The free ammonia in strictly fresh eggs varies within reasonably narrow limits and the amount of ammonia increases in an orderly fashion as the protein of the yolk deteriorates. The procedure has been used for over 20 years in the laboratory of the Massachusetts Department of Public Health as a criterion of the internal quality of liquid eggs.

The Associate Referee recommends* that the above outlined method for the determination of free ammonia in liquid eggs be studied collaboratively to the end that it may be substituted for the present tentative method.

REPORT ON DECOMPOSITION AND FILTH IN FOODS (CHEMICAL INDICES)

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

During the past year emphasis was placed on developing previously discovered chemical indicators of decomposition into form for regulatory use on specific foods. This included volatile acids, water-insoluble acids, succinic acid, indole, tryptophane—all organic compounds of relatively simple structure. No new chemical indices are proposed at this time. To establish the validity of a proposed index of decomposition in any one food, such as tuna fish, involves a thorough study of the correlation between the organoleptic condition and the numerical values of the index—a time-consuming task requiring first, the preparation of authentic packs of material, and second, analysis of hundreds of samples of the authentic material. Finally, for widespread use the method must give acceptable collaborative results. To get a chemical index of decomposition through all these stages of development requires years of elapsed time.

Perhaps the outstanding recommendation pertaining to decomposition in foods is the use of partition chromatography in the determination of the volatile acids and succinic acid. This particular type of chromatography is new in regulatory work; it adds an extra step which may seem to handicap its usefulness for routine work; however, its specificity is believed to overbalance the extra time involved, at least for those situations likely to end in court. It is hoped that a titration of a distillate at an early stage in the detailed procedure for volatile acids will serve as a "sorting test" to eliminate those non-actionable samples, so that the analyst will not have to go through the whole procedure on every sample.

Another innovation in detecting another type of decomposition, presumably not caused by microorganisms, is the use of the enzyme catecholase (also called tryosinase) as a measure of blackheart in pineapple. That most, if not all, decomposition in foods is really the result of enzymatic action is pretty well recognized; *i.e.*, where microorganisms are present their effect on food is the result of the action of the enzymes which they synthesize. In most cases it is far easier to measure the products of decomposition rather than the enzymes themselves; besides, the products are more likely to affect the organoleptic senses than would the

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 50 (1950).

enzymes which produce them. With blackheart in pineapple this condition apparently results without the aid of microorganisms. The highly insoluble dark color which is the visible indicator of decomposition is not subject to accurate measurement, whereas the enzyme catecholase, which is an essential reagent in the development of the color, can be measured with reasonable accuracy. Again, as in most of our methods of detecting decomposition, with blackheart in pineapple it is not what the nose smells or the eye sees that is measured. Of course, what is measured must correlate with what is smelled or seen if the test is to have any value.

Among predictions of future developments in this field certain more promising approaches may be mentioned. Partition chromatography will find increasing application. Paper partition chromatography will be useful particularly in the chemical detection of filth where only microgram quantities of compounds will be available. Plans for next year include, in addition to further development of chemical indices of decomposition, an active study of chemical indices of filth in foods; up to this time developments in this field have been exploratory in nature.

RECOMMENDATIONS*

The Referee concurs in the recommendations of the Associate Referees for decomposition in foods as follows:

A. Dairy products.

(1) That the method for water-insoluble acids in butter and cream be adopted as official.

(2) That the method for the determination of volatile acids in butter and cream be adopted, first action.

B. Fish and other marine products.

(1) That the official method for determination of volatile acids in fish and other marine products be modified by using the simplified distillation procedure followed by the chromatographic separation of the volatile acids; and that this modified method be adopted, first action.

(2) That the method for water-insoluble acids in fish and other marine products be studied collaboratively.

(3) That the method for succinic acid in fish and other marine products be studied collaboratively.

(4) That the method for indole in shrimp, oysters, clams, and crabmeat be adopted, first action, and further studied.

C. Eggs.

(1) That the method for water insoluble acids in eggs and egg products be adopted, first action.

(2) That the method for succinic acid in eggs and egg products be adopted, first action.

D. Fruits and fruit products.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 51 (1950).

(1) That the proposed method for catecholase activity in pineapple be studied further as a means of detecting that type of decomposition which is characterized by darkening of the fruit tissue.

(2) That the study of other products of decomposition in fruit products be pursued.

REPORT ON DECOMPOSITION IN FRUITS AND FRUIT PRODUCTS

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

BLACKHEART IN PINEAPPLE

The work on pineapple reported[1] last year indicated that the development of blackheart in the recently harvested pineapple closely paralleled the development of polyphenol oxidase (catecholase) activity in the fruit. The fresh sound fruit of low microbiological count was found to be very low in catecholase activity, while the blackheart tissue was found to possess a relatively strong catecholase activity. The method employed was a modification of the chronometric method of Nelson and Dawson[2] and included two alternative procedures. One used the time required to form sufficient *o*-benzoquinone to oxidize a known quantity of ascorbic acid. The end point was determined by siphoning the reaction solution continuously into an indicator solution of pyrogallol-starch-iodide. The other procedure was based on the color of the oxidized catechol as a measure of the enzyme activity of the sample. The procedures were not as concise, accurate, or rapid as desired. The method has since been revised and rendered concise, sensitive, and accurate. The catecholase activity is measured in fundamental units by determining the quantity of ascorbic acid oxidized in a given measured time. An excess of the ascorbic acid is used, and that remaining is measured by reaction with a given volume of the dye, 2,6-dichlorobenzenoneindophenol, along the line suggested by Ponting and Joslyn.[3] The unused dye is measured colorimetrically at a particular *p*H, and an enzyme poison is added to stop the reaction.

Stability tests under various storage conditions were made on the ascorbic acid and the dye, and it was found that these could readily be maintained for from six weeks to two months without appreciable deterioration if reagents of satisfactory quality were used. The directions for keeping the reagents were incorporated in the method. Details of the method are as follows:

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[1] *This Journal*, **32**, 511 (1949).
[2] *J. Amer. Chem. Soc.*, **63**, 3375 (1941).
[3] *Arch. Biochem.* **19**, 47 (1948).

METHOD

APPARATUS

Quartz spectrophotometer or neutral wedge photometer with filter centered at 610 millimicrons. Cells to use with instrument. 1 inch cell for photometer.

REAGENTS

(A) *Buffer soln.*—Soln 0.1 *N* each of KH_2PO_4 and $Na_2C_2O_4$. Dissolve 13.61 g of KH_2PO_4 (A.C.S.) and 13.4 g $Na_2C_2O_4$ in water, and dilute to 1 liter.

(B) *Ascorbic acid stock soln.*—Weigh exactly 200 mg of l-ascorbic acid crystals (U.S.P.), dissolve in a fresh 0.1% soln of metaphosphoric acid (C.P.), and make to 200 ml with the same solvent. Store in refrigerator (in dark) at 5°C. (40°F.) and soln will keep for month or longer.

(C) *Ascorbic acid dilute soln 1 ml = 0.1 mg.*—Dilute 10 ml of reagent (B) to 100 ml with distilled water. Soln will keep for a day.

(D)—*Standard indophenol stock soln.*—Dissolve 50 mg of Na 2,6-dichlorobenzenoneindophenol in 40–50 ml of water containing 50 mg of $KHCO_3$, shake vigorously, and when completely dissolved, dilute to 200 ml with distilled water. Store, tightly stoppered, in refrigerator at 5°C. and keep out of direct sunlight when preparing dilute soln (E). Good quality dye will keep for nearly a month, or longer To test the quality of the reagent, reduce a small amount of the soln with an excess of ascorbic acid. No red or pink color should remain in the reduced soln.

(E)—*Dilute standard indophenol soln.*—Dilute 15.4 ml (from a graduated pipet) of reagent (D) to 250 ml with water (15.4 mg/l). The soln should be standardized against the dilute ascorbic acid soln (C) under the conditions prescribed in the method below (p. 711). When 4 ml of reagent (C) are diluted to 25 ml, a 5 ml aliquot (.08 mg ascorbic) should decolorize 10 ml of the dye soln (E) almost exactly. If the two solns are not identical, discard this soln and dilute more or less of reagent (D) to obtain the strength of dye which will be equivalent to .08 mg ascorbic acid. Reagent (E) stored in refrigerator will keep for weeks.

(F)—*1.3% K_2HPO_4 – 0.25% KCN soln.*—Prepare a 2.61% soln of K_2HPO_4 and a 1% soln of KCN (1.05 g if 95% assay). Keep solns in separate bottles. Mix 10 ml of the K_2HPO_4 soln and 5 ml of 1% KCN or equivalent, and add water to make 20 ml total. This should be prepared fresh from the stock solns every 2–3 days. The KCN soln (stock) should be titrated ($AgNO_3$) at intervals to determine its strength.

(G)—*Catechol soln 1%.*—Dissolve 200 mg in 20 ml water immediately before use. (Soln sufficient for 10 detns.)

PROCEDURE

Preparation of sample.—Chop the frozen pineapple with a knife or mincer, mix, and weigh 100 g (or fraction thereof) into a tared beaker (600 ml) to an accuracy of 0.2 g. Add cold acetone until the weight taken is doubled. Stir with a glass rod for ca 30 sec. Pour the mixture into a blender, rinse in any material in the beaker with cold (5–8°C) 60% acetone (v/ v), and dilute to ca 400 ml with this latter solvent. Thoroly disintegrate the material by blending for a min. or more.

Pour the blended mixture into the original beaker, allow to drain 20–30 sec., and rinse the blender well with cold 60% acetone. Filter the mixture on an 11 cm Büchner funnel, using a Whatman No. 41 H or 54 filter paper with the aid of *gentle* suction. Allow to filter until there is no liquid above the residue and this has formed a sufficiently firm (not hard) cake so that the material will not be suspended when more liquid is added. Rinse the beaker and wash residue with about 75 ml of cold 60% acetone. When all this liquid has passed below the surface of the firm cake on the filter, wash again with about 60–70 ml of the cold 60% acetone. Allow the liquid

to suck dry (this does not mean literally dry, with no adsorbed liquid on residue), until practically no more filtrate passes thru the filter, and the residue becomes a firm, tight cake. Discard the filtrate.

Strip the filter paper from the cake of pineapple residue and transfer the latter quantitatively to a glass-stoppered graduated cylinder. Add cold distilled water until the combined volume of water and ppt. in ml is 1.5 times the weight of the sample in g (*i.e.*, make to 150 ml if 100 g sample was taken). Stopper the cylinder and shake vigorously for several min. until the pineapple residue is well dispersed.

ALTERNATE METHOD OF DISPERSING RESIDUE

Place the sample residue in a flask, add cold water equal in ml to the weight of sample taken, stopper and shake until residue is thoroly dispersed; pour the mixture into a graduate or graduated flask and rinse in any residual material with water. Make to the additional half volume, and again shake to mix well.

Pour the mixture into a centrifuge bottle and whirl at about 1500 r.p.m. for 10–12 min. Filter the supernatant liquid on a rapid (E.&D. No 195 or equivalent) filter into an Erlenmeyer flask and use for the determination of catecholase activity. Discard residue.

DETERMINATION OF CATECHOLASE ACTIVITY

Pipet 5 ml of buffer soln (reagent A) in a 25-ml glass-stoppered cylinder or a Giles flask (25–27.5 ml) and add 10 ml of the sample soln. Introduce exactly 4 ml of dilute ascorbic acid soln (reagent C), and finally add 4 ml of water to bring volume to 23 ml.

Place the cylinder (or flask) in a bath maintained at 25°C ± 0.5° for ca 15 min. Add from a rapid pipet 2 ml of reagent (G) freshly prepared. Quickly stopper and mix and simultaneously start a stopwatch or timer (within 5–8 sec.). Make to mark if necessary. Mix contents well, pipet two 5-ml portions into 1″×8″ test tubes. and place the tubes in the 25°C. bath. When exactly 5 min. have passed (from introduction of catechol), introduce 10 ml of dye soln (reagent E) from a rapid serological pipet into one of the tubes **(a)**. Blow out the last of the liquid in the pipet. Mix the contents and let stand (ca 1 min.) until the total time is 6 min. from the catechol introduction. Then add exactly 0.2 ml of K_2HPO_4-KCN soln (reagent F) from a 1-ml graduated pipet, touching the pipet to the side of the tube during the addition. Mix the tube contents well to bring the K_2HPO_4-KCN soln uniformly throughout the soln. To the other tube **(b)**, add 10 ml of water and 0.2 ml of reagent (F) and mix as above.

Read the transmission or extinction of the soln in a spectro-photometer or a neutral wedge photometer at a wave length of 610 millimicrons. Use the soln from tube **(b)** (water in place of dye) as a solvent check or standard in the spectrophotometer. If a neutral wedge photometer is used, obtain readings on both solns and subtract the reading of tube **(b)** from that of tube **(a)** to correct for the color reading of the sample soln itself.

Prepare standards by placing 5 ml of reagent (A) (buffer) in each of 5–25 ml cylinders or flasks and adding respectively 0, 1, 2, 3, and 4 ml portions of reagent (C). Add water in place of the sample solns, place in bath, and finally add 2 ml of reagent (G), catechol; make to volume, mix, and pipet 5 ml aliquots into separate test tubes. In the case of the standard No. 5 (containing 4 ml reagent (C) pipet 2–5 ml aliquots into separate tubes and reserve one of these for the addition of water.

Introduce 10 ml of the dye soln into one tube with same pipet and in the same manner as on the sample blowing out the pipet and starting a stopwatch at the beginning of the introduction of the dye. Mix quickly and when 1 min. has elapsed, add .2 ml of reagent (F) as above, and mix again.

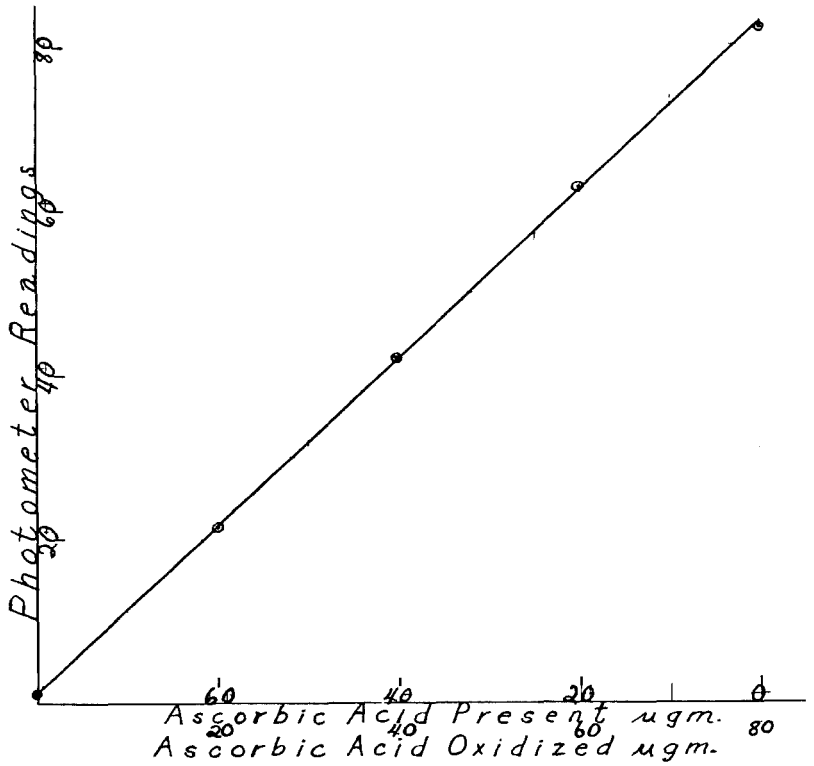

FIG. 1.—Measurement of catecholase activity.

Treat each tube in turn in this manner except the one reserved for addition of water to which is added 10 ml of water in place of dye and 0.2 ml reagent (F), etc.

Obtain the readings on these solns as described above. Readings on the neutral wedge photometer should be corrected by subtracting the difference between the reading on the soln containing water and that on same soln to which the dye was added. The fifth standard should be colorless or practically so; there should not, however, be an excess of ascorbic acid, as the amount in the aliquot (from total of 0.4 mg) should be very close to the exact equivalent of the dye soln used. A new indophenol soln (reagent E) should be prepared if there is an excess of ascorbic acid or if there is more than a minute excess of dye. This can be determined by the standard curve.

Construct a standard curve, plotting extinction coefficients or photometric readings as ordinates against ascorbic acid in mg/100 as abscissae. The scale for the amounts of ascorbic acid should be plotted from right to left, that is, with the increasing quantities of ascorbic acid toward the origin since the greater the amount of acid present, the lower the reading. Below this scale (ascorbic acid) make a second one the reverse of the first for quantities of ascorbic acid oxidized with the increasing quantities of ascorbic acid reading from left to right (Fig. 1).

From the curves and corrected reading of the sample, obtain the amount of ascorbic acid oxidized in the aliquot to which the dye was added by use of the second scale (oxidized scale).*

Multiply the quantity found by 5 (or appropriate factor) to determine the total quantity oxidized by the sample taken.

The method has been applied to a considerable number of authentic samples of pineapple which were collected last year and preserved by freezing the crushed or whole pineapple tissue. The crushed pineapple samples were preserved in sealed packages, while the whole pineapple samples were wrapped in heavy waxed paper and frozen whole. Most of the whole pineapple samples were collected from a shipment containing blackheart. They were examined by the method described above and the results are given in the accompanying Tables 1 and 2.

DISCUSSION OF DATA

An examination of the data shows that normal, sound pineapple is very low in tyrosinase (catecholase) activity, there being almost none in the newly harvested just-ripe fruit. The enzyme activity appears to increase slightly in the mellow or over-ripe fruit, but is still low. The dark material having the appearance of blackheart was a much higher tyrosinase activity, in many cases 50 to 100 times as much as the sound pineapple.

It seems probable from the data that certain types of microorganisms (such as certain bacteria and to a less extent other organisms, possibly some yeasts which cause darkening of the tissues) also produce tyrosinase (catecholase) activity. It was not determined whether or not the catecholase activity of these particular samples was due to action of microorganisms alone, or if the sample had a blackheart condition before its infection with the bacteria or other microorganisms.

The data presented give substantial evidence that the method is effective in showing those types of decomposition which cause darkening of the tissues. Although some samples which were not particularly dark showed a considerable increase in activity over the sound, nearly sterile samples, this is to be expected because the blackheart or bacterial darkening and decay of the tissue is a progressive development over a period of time.

Other examinations indicate that there is a gradual increase in catecholas activity of pineapple frozen in a sound condition over a considerable period of time; that is, a sample which previously showed an activity equal to .01 mg of ascorbic acid oxidized after 8 months storage, showed an activity of .053 mg ascorbic acid oxidized.

The writer believes that the increase in catecholase activity of these samples of frozen sound (originally) pineapple may be connected with

* NOTE 1: In the standard the greater the amount of ascorbic acid present, the lower the reading since the dye is reduced by the ascorbic acid. In the sample, however, the more ascorbic acid oxidized, the less remains and the higher the reading; the latter is therefore directly proportional to the catecholase activity.

TABLE 1.—*Polyphenoloxidase (catecholase) activity of crushed frozen raw pineapple*

SAMPLE DESCRIPTION		BLANK SAMPLE WITH H_2O	PHOTOMETER READINGS WITH DYE 0.4 mg. ADDED ASCORBIC ACID	DIFFERENCE	ASCORBIC ACID OXIDIZED IN HUNDREDTHS OF mg.
Inv. 23314K Sub A,	Fully ripe sound crushed pineapple, no sugar, 1 lb. carton, frozen.	10.6	14.3	3.7	0.8
" " Sub A_2,		10.6	14.6	4.0	1.0
Inv. 23314K Sub B,	Frozen fully ripe sound crushed pineapple, 12½ oz. fruit, 2½ oz. sugar.	8.2	9.2	1.0	0.5
" " " B_1,		11.0	16.3	5.3	1.6
Inv. 23314K Sub C_1,	Frozen slightly underripe sound crushed pineapple, packaged 1 lb. carton.	8.7	10.1	1.4	0.7
" " " C_5,		12.9	18.9	6.0	2.0
" " " C_6,		12.6	17.1	4.5	1.0
Inv. 23314K Sub D_3,	Frozen slightly underripe sound crushed pineapple, added sugar, packed 1 lb.	10.5	11.3	0.8	0.0
		13.2	20.3	7.1	2.2
Inv. 23314K Sub E_6,	Yeasty moldy or rotten unsound material (see photo) packed 1 lb. carton.	17.2	35.5	18.3	7.8
" " " E_8,		15.2	30.2	15.0	6.3
Inv. 23314K Sub F,	Commercial pack, crushed pineapple after sugar added, but before cooking.	12.5	17.0	4.5	1.8
Inv. 23314K Sub G_1,	Possible or probable blackheart	9.0	43.0	34.0	17.0
" " " G_2,	" " " "	7.8	19.8	12.0	6.0
" " " G_3,	" " " "	8.9	34.3	25.4	12.2
" " " G_4,	Probable incipient blackheart	17.0	40.5	23.5	11.2
" " " G_5,	Pineapple cores showing possible or probable incipient blackheart	5.0	12.1	7.1	3.0
" " " G_7,	Pineapple section showing possible or probable blackheart	9.2	70.8	61.6	29.2
" " " G_8,	Pineapple section similar to G_7 with probable blackheart all way to surface.	19.3	103.0	83.7*	65.6
Inv. 23318K Sub A_4,		12.7	19.0	6.3	2.1
Inv. 23318K Sub A_6,	Ripe and full yellow or possibly overripe pineapple, estery flavor.	13.2	15.1	1.9	0.0
" " " A_1,		9.2	17.9	8.7	3.3
Inv. 23318K Sub B_8,	Same as A but with added sugar, 12 oz fruit 3 oz sugar.	13.0	20.5	7.5	2.8
" " " B_1,		12.1	19.3	7.2	2.5
Inv. 23318K Sub D_2,	Sour decomposed material, mainly fermented and sour.	15.0	36.0	21.0	9.5
Inv. 23318K Sub E_1,	Otherwise decomposed material, putrified odor as well as somewhat fermented.	27.4	112.7	85.3*	199.2*

* Determinations repeated on these samples as only 40 hundreths mg ascorbic acid is usually added, since this amount reacts with 10 ml of dye soln. Larger quantities of ascorbic acid and if necessary smaller sample aliquots were used in these.

** The catecholase activity of these samples was sufficient to oxidize above 0.4 (40/100) mg in the time alloted but exact amount not determined.

TABLE 2.—*Polyphenol oxidase (catecholase) activity of raw pineapple frozen whole and taken from rejected car, containing high percentage of blackheart and other types of decomposition*

NUMBER	DESCRIPTION	PHOTOMETER READINGS		DIFFERENCE	ASCORBIC ACID OXIDIZED IN HUNDREDTHS OF mg.
		SAMPLE SOLUTION AND H_2O	SAMPLE & DYE 0.4 mg. ASCORBIC ACID		
1	Whole pineapple (sound)	6.8	7.2	0.4	0.0
2	Dissected whole pineapple, sample of dark material of appearance of blackheart	7.6	92.8	85.2	40**
3	Dissected whole pineapple, sample taken from dark portions appeared like bruise	27.0	113.7	86.7	40+**
4	Whole pineapple dissected, took sample of dark material having appearance of blackheart	43.7	129.6	85.9	40+**
5	Whole pineapple rather soft, fermented odor and inside appearance of blackheart & some secondary infection	5.2	48.5	43.3	20.8
6	Dissected whole pineapple, divided light & dark material A light, B dark like blackheart but sour smelling A B	4.8 8.9	49.0 79.7	44.2 70.8	21.6 35.0
7	Whole pineapple dissected, sample of material like blackheart appeared somewhat fermented	4.4	30.3	25.9	12.4
8		—	—	—	—
9	Whole pineapple dissected, yellowish inside sour fermented taste	8.8	52.0	43.2	21.0
10	Whole pineapple dissected, brown outside, inside greyish to light brown, very sour taste fermenting	18.5	104.3	85.8	40+**
11	Dark material from whole pineapple dissected resembling blackheart	20.8	99.8	79.0	183.6*
12	Whole pineapple dissected, typical spots like blackheart separated from light material, which appeared not affected (Light) (Dark)	6.5 17.4	11.5 104.0	5.0 87.6	1.5 55.5*
13	Whole pineapple dissected, having appearance of blackheart spots very dark separated from light material (Light) (Dark)	7.7 33.5	13.1 117.4	5.4 83.9	1.7 80+
14	Dissected whole pineapple, typical dark spots resembling blackheart reaching out from core, light & dark portions separated (Light) (Dark)	5.3 17.8	11.7 101.5	6.4 83.7	2.1 40.0
15	Dissected whole pineapple, flesh yellow, with fermented taste	8.7	17.0	8.3	3.2
16	Whole pineapple dissected, separated light and dark (Light) (Dark)	6.9 16.2	19.0 101.9	12.1 85.7	5.0 54.2*
17	Dissected whole pineapple appeared to have typical blackheart portions light separated from dark material (Light) (Dark)	5.6 19.5	15.7 106.4	10.1 86.9*	4.0 185*
18	Dissected whole pineapple, appeared quite sound but had been cut for some time	8.0	23.7	14.7	6.2
19	Dissected whole pineapple, outside soft & brown, sour fermented, flesh yellowish to brown, portion appearance blackheart, separated light and dark matter (Light) (Dark)	11.2 26.0	22.6 111.1	11.4 85.1*	4.6 80.0*

* Determinations repeated on these samples as only 40 hundredths mg ascorbic acid is usually added, since this amount reacts with 10 ml of dye soln. Larger quantities of ascorbic acid and if necessary smaller sample aliquots were used in these.

** The catecholase activity of these samples was sufficient to oxidize above 0.4 (40/100) mg in the time alloted but exact amount not determined.

the development of the "fishy" flavor often found in pineapple kept frozen for some time. The sample noted above with increased catecholase activity was beginning to develop a "fishy" flavor, and this was noted on a number of other samples. Future work should explore the range of catecholase activity of various samples of darkened pineapple and any increase in activity in sound samples after storage for varying intervals of time. Samples should also be examined for flavor, odor, and palatability at various time intervals and any changes recorded.

RECOMMENDATIONS*

It is recommended—

(1) That the method for catecholase activity in pineapple, given in this report, as a means of detecting decomposition with darkening of the tissues, be studied further.

(2) That samples of sound pineapple be frozen and portions of the samples analyzed periodically by the method; and that at the same time portions be examined for odor, flavor, and palatability, or other factors necessary to show wholesomeness of the fruit.

(3) That the study of other products of decomposition of fruits, as indicated in last year's report, be continued.

REPORT ON DECOMPOSITION IN DAIRY PRODUCTS

By FRED HILLIG (Division of Food, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

BUTYRIC ACID

At the 1948 meeting a paper[1] was presented on Volatile Acids in Cream and Butter in which it was shown that butyric acid is frequently produced in cream when it reaches that stage of decomposition where it is unfit for human consumption. The studies this year were devoted to the determination of butyric acid in butter through an application of the method of Ramsey and Patterson[2] for the chromatographic separation and determination of individual volatile acids. A separate collaborative study of the chromatographic procedure for the separation, determination and identification of volatile acids was conducted by L. L. Ramsey. A detailed report of that study is being made at this meeting (see p. 848).

To study the application of these procedures to the specific problem of the determination of butyric acid in butter, collaborators were furnished with a sample of butter prepared by a procedure designed to assure uni-

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* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 51 (1950).

[1] *This Journal*, 31, 750 (1948).

[2] *Ibid.*, 28, 644 (1945).

form composition. Twenty consecutive pound prints were collected at the creamery just off the printing line, coming from a single churn. Each pound was cut into quarters with a butter cutter and each quarter further subdivided into 20 equal-sized patties. Twenty quart jars were numbered consecutively 1 to 20 and one patty from each quarter pound print was transferred to each jar. In order to insure that the 4 patties would represent widely separated sections of the pound print, they were selected in accordance with the following scheme:

Quarter Pound No. 1

Jar No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
Pattie No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Quarter Pound No. 2

Jar No.	16	17	18	19	20	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Pattie No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Quarter Pound No. 3

Jar No.	11	12	13	14	15	16	17	18	19	20	1	2	3	4	5	6	7	8	9	10
Pattie No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Quarter Pound No. 4

Jar No.	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	1	2	3	4	5
Pattie No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20

Each collaborator was furnished with one jar of butter and a solution of butyric acid. He was requested to determine butyric acid: (1) in the butter, after preparation by the official method of sample preparation; (2) in a second sample after adding 5 ml of the butyric acid solution to one of the two 50-g portions of the butter used for the sample. The 5 ml of butyric acid solution introduced 5.3 mg of the acid to the 100 g sample of butter, which was equivalent to 6.6 mg. per 100 g of fat.

The results are given in Table 1.

The results of the collaborators on the quantity of butyric acid found in the original butter are not in perfect agreement. This may be due to the limitations of the method. The collaborators were instructed not to report a titration as butyric acid unless a definite band was discernible on the column. With butyric acid in the range of the blank reported a faint band would be obtained. Furthermore the faint band could possibly fade before it reached the outlet of the tube. In the case of the analysts who reported no butyric acid, the band may have been so faint as not to be discernible.

For the samples to which 5.3 mg of butyric acid was added by the analyst, agreement between the analysts is acceptable for determinations in the range under study.

The method follows:

DETERMINATION OF BUTYRIC ACID IN CREAM AND BUTTER

Preparation of butter sample.—Weigh 50 g into each of two centrifuge bottles and proceed as directed in the method for water-insoluble acids in cream and butter[4], beginning with the words "Add NaOH" and continuing thru the second washing with mixed ethers for removal of fat. Then remove residual ethers from bottles by evaporation on steam bath, transfer contents of both to a single 200-ml volumetric flask with H_2O, add 1 ml H_2SO_4 (1+1), and 10 ml of 10% sodium tungstate soln. Make to mark, shake, and filter. Transfer 150 ml of filtrate to distillation flask and proceed with the distillation and chromatographic separation and determination of

TABLE 1.—*Recovery of butyric acid added to butter*
(All results on fat basis)

COLLABORATOR	BLANK ON BUTTER	BUTTER WITH ADDED BUTYRIC ACID (CORRECTED FOR BLANK)	COLLABORATOR	BLANK ON BUTTER	BUTTER WITH ADDED BUTYRIC ACID (CORRECTED FOR BLANK)
	mg/100 g	*mg/100 g*		*mg/100 g*	*mg/100 g*
1	0.0	7.2	9	0.0	6.8
		7.3			6.5
2	0.9	5.8	10	0.0	8.0
		6.1			8.0
3	0.0	6.4	11	0.0	6.5
		5.8			6.2
4	2.0	5.6	12	0.0	6.9
		5.4			7.1
5	0.0	5.6	13	0.9	6.0
		5.8			5.5
6	1.6	6.0	14	0.9	6.4
					7.4
7	1.3	6.2	15	0.0	7.8
					6.8
8	1.3	5.9	16	1.8	6.1
		6.0			6.0

butyric acid as directed in the determination of volatile acids in fish. (Chromatographic procedure adopted first action, at the 1949 meeting.)*

Preparation of cream sample.—Weigh 100 g into a 250-ml volumetric flask, add 100 ml of water and 2 ml of H_2SO_4 (1+1), and mix, avoiding violent shaking Add 15 ml of 10% sodium tungstate soln, make to mark, shake and filter thru a rapid flowing filter paper. Transfer 150 ml of filtrate to distillation flask and proceed as above for butter.

WATER-INSOLUBLE FATTY ACIDS (WIA)

Last year the method for the determination of water insoluble fatty acids (WIA) in cream and butter, was submitted for collaborative

* Methods of Analysis, 7th Ed., 1950.

study.[3] On the basis of the satisfactory results reported the method was adopted as first action.[4]

This year three samples of butter in duplicate were again submitted for collaborative study. The collaborators were not advised of the fact that they received duplicate samples and were asked to make single determinations on each of the six samples received. The results are given in Table 2.

TABLE 2.—*WIA in collaborative butter samples—1949*

COLLABORATOR	SAMPLES A, D		SAMPLES B, E		SAMPLES C, F	
	MG/100G FAT	MMW*	MG/100G FAT	MMW*	MG/100G FAT	MMW*
1	474	260	344	259	794	263
	472	266	330	258	794	263
2	478	269	338	271	796	266
	482	267	328	267	—	—
3	468	268	317	265	769	270
	469	270	334	267	782	270
4	484	285	355	278	779	275
	486	285	329	286	779	275
5	399	272	318	277	764	276
	413	275	306	274	763	278
6	423	277	366	281	800	277
	439	274	340	272	819	284
7	459	273	355	264	793	269
	462	271	355	273	796	268
8	470	268	330	261	776	266
	467	266	326	262	777	267
9	387	269	386	269	858	266
	389	271	388	271	848	266

* Mean molecular weight.

The data obtained by analysts No. 6 and No. 9 on samples A and D are somewhat low when compared to those obtained by the other collaborators. Also, the data obtained by analyst No. 9 on samples C and F appear to be somewhat high. With these few exceptions the results are very satisfactory. The mean molecular weights, which for the most part fall within the range for palmitic, oleic, and stearic acids (the principal higher

[3] *Ibid.*, 32, 520 (1949).
[4] *Ibid.*, 32, 91 (1949).

molecular weight fatty acids present in butter as glycerides), indicate that the acids are relatively free of non-acidic material.

RECOMMENDATIONS*

It is recommended—

(1) That the method for the determination of butyric acid in cream and butter be adopted, first action.

(2) That the method for the determination of water insoluble fatty acids in cream and butter[4] be adopted as official.

Grateful appreciation is extended to the following collaborators, all members of the Food and Drug Administration, who participated in the work:

H. P. Bennett, New Orleans
Helen C. Barry, New Orleans
S. D. Fine, Cincinnati
H. C. Van Dame, Cincinnati
Fred Garfield, St. Louis
Mary McEnery, St. Louis
A. Carson, St. Louis
L. W. Ferris, Buffalo
J. W. Welch, Buffalo
H. P. Eiduson, Buffalo
L. E. Wener, Chicago
C. A. Wood, New York
L. C. Mitchell, Minneapolis
H. W. Conroy, Kansas City
Catherine C. Cunningham, Boston
H. M. Boggs, Philadelphia
Shirley M. Walden, Baltimore
H. O. Fallscher, Seattle
H. M. Bollinger, Los Angeles
R. E. O'Neill, Atlanta
A. Kleinman, San Francisco

REPORT ON DECOMPOSITION IN FISH

By Fred Hillig (Division of Food, Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Associate Referee*

VOLATILE ACIDS

The distillation procedure for volatile acids in fish has been accepted by this Association as official and appears in the 1945 *Book of Methods*, (**24.9** to **24.11**). Subsequent to the adoption of this method by the Association, a chromatographic procedure for the separation and identification of the isolated volatile acids was proposed.[1] Since this procedure is more specific than computation of the acids based on their rate of distillation under controlled conditions, as called for in the present official method, it was recommended at the meeting last year that a simplified distillation procedure followed by chromatographic separation of the isolated volatile acids be studied collaboratively. Since the apparatus and distillation procedure for the isolation of the volatile acids in the official method have not been materially changed, it was felt that this step need not be studied collaboratively this year. Therefore, the study was devoted to the chromatographic separation and determination of the volatile acids, starting with their water solutions. For a report on this collaborative study, see

[1] *This Journal*, **28**, 644 (1945).

p. 848. The method used is given in detail in *Methods of Analysis*, 7th Ed., 1950.

WATER-INSOLUBLE ACIDS (WIA)

In the report made last year it was stated that preliminary work on WIA in fish and fish products indicates that this way may be another method for detection of the use of decomposed material. This work has been extended during the past year to the authentic packs of fish and fish products that were available, and from the results it would appear that this determination will be of value for some products.

Succinic acid: Further work on the determination of succinic acid in fish and fish products indicates that this determination also may be another measure of decomposition.

RECOMMENDATIONS*

It is recommended that—

(1) The official method for determination of volatile acids in fish and other marine products be deleted.

(2) The simplified distillation procedure attached to this report, followed by the chromatographic separation of the volatile acids, be adopted, first action.

(3) The simplified distillation procedure followed by the chromatographic separation of the volatile acids be further studied collaboratively, with a view to its adoption as official.

(4) The method for water-insoluble acids be studied collaboratively.

(5) The method for succinic acid be studied collaboratively.

REPORT OF DECOMPOSITION IN SHELLFISH

By R. E. DUGGAN (Food and Drug Administration, Federal Security Agency, New Orleans, Louisiana), *Associate Referee*

Subcommittee C[1] recommended that the method for Indole in Shrimp, Oysters, and Crabmeat[2] be adopted as a tentative method. Collaborative results were submitted before adoption as a tentative method; and since no reports indicating a need for changes in the method have been received it was not felt that additional collaborative studies were necessary. However, in the course of the continuing search for new indices of decomposition in shellfish, the indole content of shrimp at varying stages of decomposition have been determined under the supervision of the Associate Referee by other chemists. The shrimp were allowed to spoil under controlled conditions and each shrimp was classified organoleptically[3] by the

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[1] *This Journal*, **31**, 51 (1948).
[2] *Ibid.*, **31**, 96 (1948).
[3] *Ibid.*, **29**, 177 (1946).

Associate Referee and others. The following tabulation presents the results:

TABLE 1.—*Indole in shrimp*
mmg/100 g

ANALYST	H. P. BENNETT			H. P. BENNETT			J. E. ROE AND M. J. GNAGY
EXPERIMENT	A			B*			C
PRODUCT	*Raw*	*Blanched*	*Canned*	*Raw*	*Blanched*	*Canned*	*Raw*
Class 0	0.5	0.3	0	4.2	4.6	—	1.6
Class 1	5.5	5.7	3.5	1.5	5.9	4.0	2.7
Class 2	50.9	20.9	51.8	8.4	15.6	9.3	12.5
Class 3	109.6	259.6	243.4	41.0	47.8	60.0	172.0

* Ammoniacal type of decomposition.

These data are similar to those obtained in earlier experiments.[3]

The Associate Referee observed that the detailed procedure was followed by these chemists with no apparent difficulties. The Associate Referee has also observed other chemists using this method with success in the laboratory of the New Orleans District for two years in the determination of indole in shrimp and oysters.

Studies have been instituted to determine the usefulness of substances such as water-insoluble acids, formic acid, succinic acid, and free tryptophane, as indices of decomposition in shrimp. It is not possible to judge the usefulness of these substances as indices of decomposition on the basis of the data available at this time; therefore the data are not being presented.

It is recommended*—

(1) That the method for indole in shrimp, oysters, and crabmeat be adopted, first action.

(2) That studies be continued on the investigation of substances suitable for detecting decomposition in shellfish.

REPORT ON DECOMPOSITION IN EGGS

By FRED HILLIG (Division of Food, Food and Drug Administration, Federal Security Agency, Washington 25, D. C.), *Associate Referee*

SUCCINIC ACID

The proposed method for succinic acid in eggs is based on an ether extraction of the acid from a clarified water extract of the egg product and subsequent separation of the succinic acid on a chromatographic column.[1] In another report[2] the method was applied to edible shell eggs secured

* For report of Subcommittee C and action of the Association see *This Journal*, **33**, 51 (1950).
[1] H. V. Claborn and W. I. Patterson, *This Journal*, **31**, 134 (1948).
[2] Henry A. Lepper and Fred Hillig, *Ibid.*, **31**, 734 (1948).

from various sections of the United States; no succinic acid was found. In an authentic pack of dried eggs it was also shown that succinic acid was present in those samples prepared froum sour liquid eggs.

For the collaborative study of this method, three samples of dried eggs were distributed; the results are given in Table 1.

TABLE 1.—*Succinic acid in dried eggs*
mg/100 g

COLLABORATOR	SAMPLE 1	SAMPLE 2	SAMPLE 3
1	None	44.2	127.4
		46.6	122.7
2	None	49.6	115.2
		48.4	130.8
3	None	42.0	98.4
		42.7	101.5
4	None	47.6	129.2
		47.2	130.0
5	None	44.1	137.7
		53.2	117.6
6	None	41.3	100.0
		41.9	98.2
7	None	52.8	130.0
		54.0	129.0
8	None	42.1	111.0
		41.4	104.5

Sample 1 was prepared from edible eggs and no succinic acid was found by any of the collaborators. The liquid egg magmas from which samples 2 and 3 were prepared were sour before drying. Succinic acid was present in both of these samples. Duplicate determinations were satisfactory, as were the checks between the various collaborators.

In order to get collaborative data on the reproducibility of the method on liquid eggs, which are difficult to ship, 2 chemists from each collaborating laboratory were instructed to break out edible eggs and to immediately start the determinations of succinic acid in duplicate in the magma. The magma was then allowed to stand until it became sour, when succinic acid was again determined. The data are given in Table 2.

No succinic acid was found by any of the collaborators in the edible eggs used in the study, while in every case except one the acid was found in the sour eggs. This single result is surprising, but of course the bacteria

TABLE 2.—*Succinic acid in liquid eggs*
mg/100 g

COLLABORATING LABORATORY	ANALYST	FRESH EGGS	SOUR EGGS
1	A	None	13.9
			12.7
	B	None	13.7
			13.4
2	C	None	None
	D	None	None
3	E	None	2.9
			4.5
	F	None	2.2
			2.6
4	G	None	13.9
			13.3
	H	None	15.2
			15.5
5	I	None	39.2
			38.8
	J	None	—
			40.4
6	K	None	25.8
			25.8
	L	None	26.2
			25.8
7	M	None	19.4
			19.1
	N	None	18.0
			19.7
8	O	None	36.9
			36.4
	P	None	27.2
			34.8

present in each egg batch were undoubtedly different. It is most likely that those causing the souring in this one instance were not of a species which forms succinic acid during the decomposition of the egg material. Obviously there is no relation between the quantities of succinic acid in the different magmas. Therefore, the judgment of acceptability of the method for adoption is based upon the concordance of the duplicate re-

TABLE 3.—*Water insoluble fatty acids in eggs*
mg/100 g

COLLABORATOR	SAMPLE 1		SAMPLE 2		SAMPLE 3	
1	690		5,000		8,265	
	680		4,925		8,125	
2	825	705	5,270	5,040	7,780	7,175
	815	600	4,705	4,625	7,770	7,740
3	661		5,734		9,557	
	664		5,610		9,679	
4	895		5,615		10,740	
	890		5,740		10,165	
5	745		5,525		8,190	
	550		4,935		7,545	
6	255		3,150		6,815	
	235		3,610		6,810	
7	640		5,208		8,506	8,612
	674		5,526		9,297	8,532
	684		4,778			
8	650		5,580		8,950	
	685		5,550		8,980	
9	885		5,590		9,465	
	805		6,120		9,130	
10	880		5,900		10,285	
	665		6,440		10,465	

sults obtained by each analyst and the agreement between each pair of analysts working on the same eggs.

The original method for the determination of succinic acid in eggs and egg products[1] was modified in some details. This modified procedure appears in the 7th Ed., *Methods of Analysis* (1950).

WATER-INSOLUBLE FATTY ACIDS

In a previous report[4] the method for the determination of water-insoluble fatty acids (WIA) in cream and butter[5] was adapted to dried eggs. Excellent recoveries were obtained when a mixture of oleic and palmitic acids was added to dried eggs. Analysis of an authentic pack of dried eggs showed that when the liquid eggs were sour the quantity of W.I.A. was materially higher than that found in the dried product from edible liquid eggs.

[4] Fred Hellig, *This Journal*, 31, 731 (1948).
[5] *Ibid.*, 30, 575 (1947).

Since the data indicated that WIA is an index of decomposition in eggs, the method[4] was studied collaboratively. However, it has not been demonstrated that increase in WIA occurs in all types of decomposition in eggs. Three samples of dried eggs were submitted to the collaborators; the results are given in Table 3.

The collaborative results are in good agreement, with the exception of analyst 6, whose results are consistently low. No reason can be assigned by this analyst in explanation. In the light of the interpretation to be placed on the quantities of WIA found, low results by one collaborator are not regarded as significant in the evaluation of the method for adoption. The concordant results by all the other analysts support the following recommendation: It is recommended that the method for the determination of succinic acid in eggs and egg products be adopted, first action.

It is recommended that the method for the determination of water-insoluble acids in eggs as described in the previous report[4] be adopted, first action.

Grateful appreciation is extended to the following collaborators, all members of the Food and Drug Administration, who participated in this work:

L. C. Mitchell, Minneapolis
Sam H. Perlmutter, Minneapolis
L. W. Ferris, Buffalo
H. P. Eiduson, Buffalo
H. C. Van Dame, Cincinnati
F. J. McNall, Cincinnati
M. L. Dow, St. Louis
H. D. Silverberg, St. Louis
N. A. Carson, St. Louis
Mary A. McEniry, St. Louis
H. I. Macomber, Baltimore
Shirley W. Walden, Baltimore
L. H. McRoberts, San Francisco
D. W. Williams, San Francisco
G. Kirsten, New York
Helen C. Barry, New Orleans
S. Kahan, New York
Sidney Hess, Division of Food, Washington, D. C.

VOLATILE ACIDS

The present procedure for the determination of volatile acids in eggs is based on a computation of the acids from the data obtained on their rates of distillation under controlled conditions (sec. **24.10, 25.11**).

A new chromatographic procedure has been adopted for the separation and determination of volatile acids (see p. 707). The acids will first be isolated by distillation from the food. Since the acids will not be determined by computation, the standardization of the distillation apparatus, sec. **24.10**, will be simplified. This procedure is applicable to the volatile acids distilled from eggs. Since it is more specific than the present official method, it is recommended that Sec. **23.36** and **23.37** be deleted and the simplified distillation procedure, followed by the chromatographic separation of the volatile acids, as referred to the report on decomposition in fish (see p. 720) be adopted, first action. The revised method will appear in *Methods of Analysis*, 7th Ed., 1950.

RECOMMENDATIONS*

It is recommended—

(1) That the method for succinic acid in eggs and egg products be modified and made first action.

(2) That the method for water-insoluble acids in eggs and egg products be made first action.

(3) That the method for the determination of volatile fatty acids be modified according to the new chromatographic technic and made first action.

A paper entitled "A Collaborative Study of the Chromatographic Separation and Determination of Volatile Fatty Acids," by L. L. Ramsey and S. M. Hess, is given on p. 848.

REPORT ON GELATIN, DESSERT PREPARATIONS, AND MIXES

By S. C. Rowe, (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Referee*

In accordance with last year's recommendations of the General Referee, the methods for jelly strength (paragraphs **9.6** and **9.12**), sucrose, and dextrose (paragraphs **9.13** and **9.15**) were studied collaboratively during the year. We were very fortunate in securing the services of Paul A. Kind of the Kind and Knox Gelatin Co., Camden, New Jersey, and Joseph H. Cohen, General Manager, Atlantic Gelatin Division, General Foods Corporation, Woburn, Massachusetts, as Associate Referees. Dr. Kind, who was unable to be at the meeting, was represented by Dr. D. Tourtellotte.

Samples of different jelly strength were submitted to eleven collaborators in the case of plain gelatin, and twelve collaborators in the case of gelatin desserts. Most of the collaborators completed their work and reports were submitted. The samples were accompanied by special plungers for use with the Bloom Gelometer. In the case of plain gelatin a full half-inch plunger with sharp edges was used, since it was found that rounding of the edges had taken place on many of the plungers currently being used on Bloom Gelometers. The plane surface in contact with the test sample had been reduced in area, in extreme cases by as much as 32% of a full 0.5 inch plunger. In the case of gelatin desserts attempts have been made from time to time to slow down the flow of shot, the speed of operation being too great for accuracy and reproducibility. The present method has a shot-flow regulator which has not proven entirely satisfactory, for the reason that it will not regulate the shot flow uniformly from one instru-

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 51 (1950).

ment to another and cannot be applied in exactly the same manner to a given instrument at different times. The Associate Referee suggested a larger plunger, 1.0″ in diameter, maintaining other conditions and adjustments on the same basis as for the determination of jelly strengths of gelatins. The names "standard A.O.A.C. 1.0" and "standard A.O.A.C. 0.5 inch" have been suggested for these new plungers.

The collaborative results obtained on plain gelatin were in very close agreement. The new 0.5 inch diameter plunger has shown remarkable reproducibility, even in the lower strength gelatin, where it was feared there might be some cutting of the surface of the gelatin. The larger 1.0 inch diameter plunger for gelatin desserts gave a higher jelly strength value and a more significant spread over the range of samples than did the smaller plunger. The report clearly shows this in the accompanying graphs. The report also contains additional data from the Swift & Co. laboratories supporting the Associate Referee's recommendation that the 1.0 inch plunger be adopted for gelatin desserts.

RECOMMENDATIONS*

The Associate Referee has recommended certain changes in the methods under paragraphs **9.6** and **9.12**, and in addition has recommended the use of the 0.5 inch and 1.0 inch diameter plungers for gelatin and gelatin desserts. Drawings of these plungers have been submitted. The General Referee concurs in these recommendations and also recommends that the description of the methods be accompanied by the sketches of the plungers so that there will be no doubt as to their dimensions.

The Associate Referee on Gelatin Dessert Constituents sent out samples of gelatin desserts to five collaborators for a determination of sucrose and dextrose. The results obtained showed that the reproducibility was good. No attempt was made to explore the accuracy of the method, which made it unnecessary to have samples of known composition. He has recommended that the present methods for sucrose and dextrose, paragraphs **9.13** and **9.15**, be made first action, and that collaborative work be conducted next year with samples of known composition. The General Referee concurs in this recommendation.

In considering the other methods as they now appear in the 6th Edition, many of them are already official methods for other products. Where the directions call for the use of a well-known official method by reference to another chapter as, for example, in the determination of moisture under paragraph **9.2**, and no difficulty is experienced in the use of the method, it is believed such a method should be made official. Accordingly, it is recommended that paragraphs **9.2–9.5**, inclusive, **9.8–9.10**, inclusive and **9.17–9.19**, inclusive, which are methods for moisture, ash, total phosphorus, and nitrogen, be made official.

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 52 (1950).

The methods for starch in Starch Dessert Powder (paragraph **9.21**) have not been studied collaboratively, but since these are standard methods by reference to other chapters in the *Book of Methods*, it is recommended that they be made first action.

Recently it has been found that the method for Total Acidity (paragraph **9.11**) gave erratic results, because of the small size of the sample. There was a considerable error due to segregation, when the sample consisted of only 1 gram. The work performed by the Kind and Knox Co. showed that the error decreases from about 30% with a 1-gram sample to about 2% with a 30-gram sample. The work of the New York District of the Food and Drug Administration demonstrated the necessity for a revision of the method (comparing 1-gram samples and 20-gram samples). It is recommended that paragraph **9.11** be revised to read as follows, and that it be adopted as first action.

"Dissolve 20 grams of material in 2 liters of recently boiled H_2O. Titrate 100 ml with 0.1 *N* NaOH using 0.3 ml of 1% phenolphthalein solution, 2.10(d). Report as percentage by weight of citric acid."

A contributed paper entitled "Water-Insoluble Fatty Acids and Butyric Acid in Cream and Butter," by Fred Hillig, H. A. Lepper, and W. I. Patterson, was published in *This Journal* November, 1950.

REPORT ON JELLY STRENGTH METHODS

By DEE TOURTELLOTTE and PAUL A. KIND (Kind and Knox Gelatin Co., Camden, N. J.), *Associate Referees*

SECTION I. GELATIN

The Bloom Gelometer[1,2] has become the standard instrument for the determination of jelly strength values of gelatin and gelatin dessert preparations, in the industry and by various regulatory and purchasing organizations.

Certain unspecified and untested changes have been permitted to creep in by various users of the instrument until it has been incriminated as an unreliable method of testing. As a result of discussions with members of the Revision Committee of the A.O.A.C., regarding the necessity for additional study of the tentative jelly strength methods, the Referee, S. C. Rowe, of the Food and Drug Administration, recommended appointment of Paul A. Kind of the Kind and Knox Gelatin Company, Camden, New Jersey, as an Associate Referee to conduct collaborative tests and make recommendations for an official method of jelly strength tests on gelatin and gelatin dessert powders.

[1] National Association of Glue Manufacturers, *Ind. Eng. Chem.*, **16**, 310 (1924).
[2] ——, *Ind. Eng. Chem.*, Anal. Ed., **2**, 348 (1930).

An appraisal of the tentative method **9.6** (Sixth Ed., 1945) showed an error in the preparation of the sample; and a survey of Bloom Gelometers in use showed that a varying amount of rounding of the edges of the plunger had been made, until the plane surface in contact with the test sample had been reduced in area, in extreme cases, by as much as 32 per cent of the full 0.5-inch plunger.

Quoting, from the original report on the adoption of the Bloom Gelometer, in "Industrial and Engineering Chemistry," Vol. 16, Page 315 (March 1924) it is stated as follows:

"(G) *Effect of Plunger Area.* Other things being equal, the jelly test increases with increase in the area of the plunger. For this reason it is essential that the diameter of the plunger be standardized. The Committee recommends that the standard plunger of the Bloom Gelometer be circular in cross section, and 12.7 mm. (0.5 inch) in diameter."

It seems desirable now to set a plunger specification which can be regarded as standard for the instrument. Since the original method called for a full half-inch plunger with sharp edges, it was decided with the permission of the Referee to make a number of accurate plungers and distribute one to each collaborator with the test samples.

For the purpose of the collaborative test the tentative method was rewritten as follows, to coincide with practice:

"Add 7.5 g of sample to 105 ml of water at 10° to 15°C., measured from pipette, in standard Bloom bottle, followed by stirring. Let stand for one hour and then bring to 62° in 15 min. by placing in a water bath regulated at 65°; the sample may be swirled several times to aid solution. Finally mix by inversion, let stand 15 min., and place in a water bath controlled at 10°C. ±0.1°. Chill for 17 hours. Determine jelly strength on Bloom Gelometer (2) adjusted for a 4 mm depression and to deliver 200 g shot/5 seconds (± 5 g) using the 0.5″ plunger."

A letter of transmittal, together with method and report blanks, test samples, and an accurate plunger, were distributed to the following list of collaborators:

M. H. Merchant, Amer. Agric. Chem. Co., P.O. Drawer 2458, Detroit, Mich.

Joseph H. Cohen, General Foods Corp., Atlantic Gelatine Div., Woburn, Mass.

R. G. Buchner, Milligan & Higgins, 222–224 Front Street, New York 7, New York.

H. Epstein, Grayslake Gelatin Co., Grayslake, Ill.

Frederick C. Minsker, Food and Drug Administration, 1204 New Custom House, Philadelphia 6, Pa.

M. D. Given, Eastman Gelatin Corporation, Peabody, Mass.

F. L. DeBeukelaer, Swift & Company, Union Stock Yards, Chicago, Ill.

Sumner C. Rowe and Edward Steagall, Food and Drug Administration, Washington, D. C.

Thomas B. Downey, Kind & Knox Gelatin Co., Camden, N. J.

TABLE 1.—*Summary of collaborative jelly strength tests*
Revised method **9.6**, using tentative A.O.A.C. 0.5″ plunger

LABORATORY	HIGH TEST AV. 3 DETN.		MED. TEST AV. 3 DETN.		LOW TEST AV. 3 DETN.	
A	267		195		72	
	273		198		72	
	273		201		71	
Av. 9 Detn.	272	(264)*	198	(192)*	72	(71)*
B	266		196		70	
	268		198		70	
	267		198		71	
Av. 9 Detn.	267	(253)*	197	(189)*	70	(67)*
C	267		201		70	
	269		198		71	
	273		191		72	
Av. 9 Detn.	269	(256)*	197	(192)*	71	(68)*
Group ave.	269		197		71	
D	275		201		69	
	275		199		71	
	274		199		68	
Av. 9 Detn.	275		200		69	
E	281		204		73	
	283		205		73	
	279		200		71	
Av. 9 Detn.	281		203		72	
F	284		207		76	
	284		209		73	
	281		206		75	
Av. 9 Detn.	283		207		74	
G	281		205		75	
	280		203		74	
	281		205		75	
Av. 9 Detn.	281	(254)*	204	(187)*	75	(70)*
H	280		205		73	
	280		205		71	
	281		204		71	
Av. 9 Detn.	280	(263)*	204	(195)*	72	(69)*
I	281		205		68	
	278		201		69	
	282		205		68	
Av. 9 Detn.	280		204		68	
Group ave.	280		204		72	
Total ave.	276		201		71	

* Results with regular gelometer in current use. For comparative purposes only.

C. B. Streightif, United Chem. & Org. Products, Chicago, Ill.

The summary of the results reported by the respective collaborators is given in Table 1 and shows reasonably good agreement, considering that this was only a single trial. Without subjecting the results to any exacting analysis the majority opinion of the collaborators was approval of the method as rewritten, and also approval of the full 0.5 inch plunger which we have chosen to call the A.O.A.C. Bloom Gelometer 0.5 inch plunger.

Practically the only objection to the new plunger was the possibility that it would cut the surface of weak jellies and give erratic results. Actually, the new 0.5 inch A.O.A.C. plunger has shown excellent reproducibility with the lower strength gelatin jellies, thus tending to answer that objection.

The "General Precautions" for jelly strength determination with the Bloom Gelometer as given in the report by DeBeukelaer *et al.*[3] must be emphasized again in these tests, since some of the errors in the final results must be attributed to variations in these factors of control.

"(F) General Precaution:

As a result of several years of testing experience using the above-described equipment and methods, the committee wishes to emphasize the following general precautions:

1. *General:*

(a) Check all balances used.

(b) Check of soaking and melting procedures.

(c) Check all thermometers, especially those used in chill bath and viscosity determination. Thermometers furnished for precision work are sometimes found to be inaccurate to as much as 0.5 degrees. Therefore, all should be checked for accuracy against a reliable standard.

2. *Jelly Strength:*

(a) Check of gelometer prior to every series of tests for correct rates of shot flow (200 grams ± 5.0 per five seconds). With continued use the shot wears unevenly, thus affecting the rate of flow. It should therefore be renewed from time to time.

(b) Frequent check of 4-mm. gap between contact points.

(c) Proper leveling of gelometer.

(d) Condition of batteries.

(e) Maintenance of correct temperature of bath during chilling period.

(f) Uniformity of temperature throughout the bath.

(g) Checking of jell surface (foam, unevenness, etc.).

(h) Keep shot for gelometer clean, dry, and well protected in a closed container when not in use."

For example, some collaborators reported difficulty in maintaining constant temperature in the chill bath, others had assumed the accuracy of the 4-mm. contact adjustment, and a later check showed that the tests had been run with approximately a 5 mm. gap. The skill of the operator in making the fine adjustment at the starting point of each test, so as to avoid an initial loading of the sample beyond the two to three gram spring displacement, is an extremely important factor.

[3] *Ind. Eng. Chem.*, Anal. Ed., **2**, 351 (1930).

RECOMMENDATIONS*

It is recommended—

(1) That the tentative method as rewritten and tested using the standard Bloom Gelometer with the A.O.A.C. 0.5″ plunger having full 0.5″ plane surface, be adopted as the official jelly strength method to replace method 9.6 (*Methods of Analysis*, A.O.A.C., sixth ed.)

(2) That the 0.5″ plunger as detailed in Figure 1 be adopted as the standard A.O.A.C. Bloom Gelometer plunger, for the jelly strength test of gelatin.

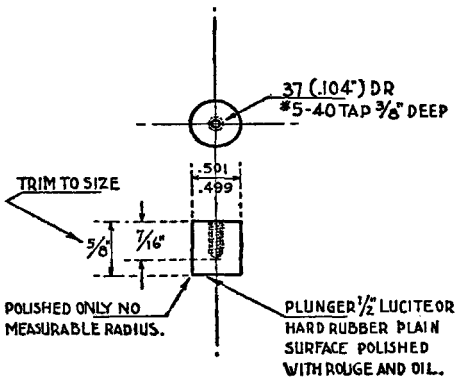

FIG. 1.—0.5-inch Bloom Gelometer Plunger.

SECTION II. GELATIN DESSERT POWDER

In the past the use of the Bloom Gelometer as adopted for the determination of the jelly strength of gelatin has been applied without special modification to the determination of jelly strength of gelatin dessert powders.

The instrument has inherent characteristics which lead to a marked degree of error in the testing of weak jellies as best summarized in a statement quoted from F. L. DeBeukelaer, O. T. Bloom, and J. T. DeRose,[4] as follows:

"The Bloom gelometer has one fault which causes concern when testing weak jellies, such as gelatin dessert jellies. In such cases, when the standard procedure is followed, the operator hardly has time to release hold of the arm of the shot-flow device before the cut-off mechanism functions. This fact, together with the suddenness of the application of the load to the jelly surface, results in the addition of an excess, due probably to the inertia of the jelly to deformation. Finally, there is the usual error due to the shot still in mid-air at the instant of cut-off. The modified method given below reduces these errors to a practical minimum."

In the above reference an accessory shot-flow regulator is proposed for

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 52 (1950).
[4] *Ind. Eng. Chem.*, Anal. Ed. 17, 64 (1945).

slowing the rate of loading and thus avoiding in principle some of the difficulties referred to. Experience has shown, however, that the adapter as recommended will not regulate the shot-flow uniformly from one instrument to another and cannot be applied in exactly the same manner to a given instrument at different times.

Experience in the laboratory of the Associate Referee showed the feasibility of using a larger plunger, 1.0″ in diameter, on the Bloom Gelometer and maintaining other conditions and adjustments on the same basis as for the determination of the jelly strength of gelatins. Accordingly, jelly strength method **9.12** (*Methods of Analysis*, A.O.A.C. sixth ed.) was rewritten incorporating the use of a "standard A.O.A.C. 1.0″ plunger." Collaborative comparative tests were run with this plunger versus the "A.O.A.C. 0.5″ plunger." The method, varying only in the plunger specification, is as follows:

To 20 g of powder in standard Bloom bottle, add from pipette, with stirring, 100 ml of water at 10–15°C. Let stand for 15 min. and then bring to 62° in 15 min. by placing in water bath regulated at 65°; the sample may be swirled several times to aid solution. Finally, mix by inversion, let stand 15 min. and place in water bath controlled at 10° ±0.1°. Chill for 17 hours. Determine jelly strength on Bloom Gelometer (2) adjusted for a 4-mm depression and to deliver 200 gm shot/5 seconds (± 5 gm) using the 1.0″ plunger and light weight shot receiver (paper or plastic).

A number of accurately made plungers (see Figure 2) were prepared and distributed with method, report blanks, and test samples, to the following collaborators, in addition to those named previously:

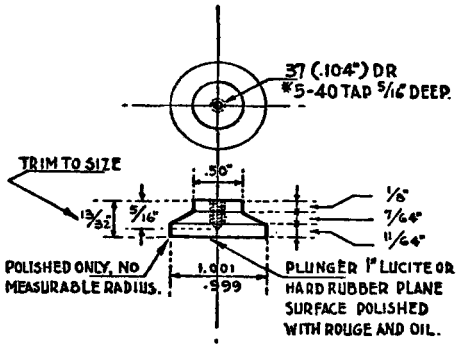

FIG. 2.—1.0 inch Bloom Gelometer Plunger.

E. J. Pelaccio, Standard Brands, Peekskill, New York

J. S. Kujawski, Lt. Colonel, Department of the Army, Quartermaster Food & Container Inst., 1849 West Pershing Rd., Chicago 9, Ill.

Norman F. Armstrong, Charles B. Knox Gelatine Co., 4th and Erie Streets, Camden, N. J.

The results of individual collaborators are reported in Table 2.

TABLE 2.—*Collaborative results*

LABORATORY	SAMPLE NO. 1		SAMPLE NO. 2		SAMPLE NO. 3	
	1.48% GELATIN		1.58% GELATIN		1.95% GELATIN	
	0.5"	1.0"	0.5"	1.0"	0.5"	1.0"
A	19.0	47.3	21.0	51.7	26.7	92.3
	17.7	47.3	21.0	52.0	27.3	93.0
B	16.0	33.0	18.0	38.0	26.0	77.0
	17.0	32.0	18.0	39.0	25.0	76.0
C	14.7	33.7	17.0	39.3	22.0	71.7
	15.7	33.0	17.0	38.6	22.0	70.7
D	15.7	35.0	—	44.2	22.2	77.5
E	13.6	33.2	15.7	36.3	23.8	66.2
	13.7	32.2	16.0	35.8	24.2	65.5
F	11.0	40.6	12.0	46.0	19.6	85.0
	11.3	40.3	11.6	45.6	19.0	84.6
G	18.0	36.6	20.3	44.6	26.6	76.6
	17.0	38.3	20.6	45.6	27.6	78.3
H	16.0	37.0	18.0	43.0	26.0	81.0
	16.0	37.0	18.0	43.0	25.0	79.0
J	23.8	37.5	24.7	44.7	27.5	83.7
	24.0	35.0	24.7	42.2	26.3	82.3
K	18.3	36.6	18.7	39.8	24.8	73.2
	17.7	35.0	18.5	40.1	25.6	72.6
Average	16.6	36.7	18.4	42.6	24.5	78.1

Comparison of results from the use of the 0.5" and the 1.0" plungers shows a relatively higher jelly strength value and a more significant spread over the range of samples when tested with the larger plunger. This is more clearly brought out in Figure 3 where the average results are plotted. The very flat curve resulting from the 0.5" plunger, over a rather wide range of gelatin concentrations, explains how even slight errors in the procedure can lead to difficulties, because slight differences in jelly strength are impossible to detect.

The steep slope of the curve resulting from the 1.0" plunger with the same test samples indicates that manipulative errors limit the jelly strength variations to a much smaller range. The fact that the curve, relating Bloom grams to gelatin concentration when tested with the 1.0"

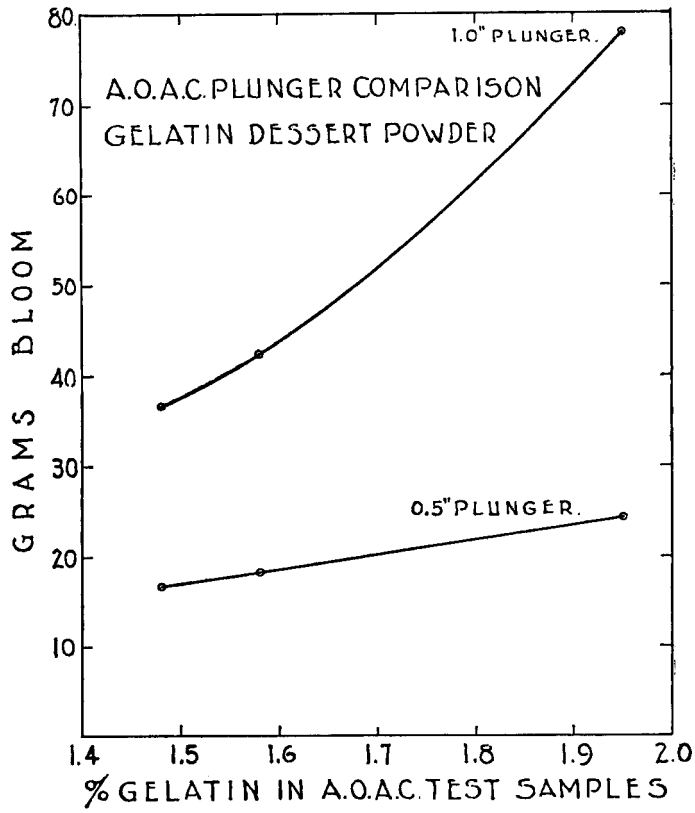

FIG. 3.—Average Bloom values of A.O.A.C. gelatin dessert test samples using 1.0" and 0.5" plungers.

plunger, is not a straight line is due to an altered displacement factor resulting from the change in ratio of areas of plunger and test sample. By specifying the standard Bloom bottle with the 1.0" plunger this ratio will be a constant and actually increases the sensitivity of the test.

It would be necessary to make a much larger series of determinations over the approximate range represented by these A.O.A.C. test samples in order to formulate a more accurate relationship of jelly strength and gelatin concentration when using the 1.0" plunger, and thus determine an average mean variation at any point on the curve.

There are other factors, such as *p*H of the gelatin dessert, and the type and jelly strength of the gelatin used, which of necessity would have to be taken into account in any further research on this problem.

Additional data has been contributed by the Swift Laboratories which supports the experience of the Associate Referee's laboratory in rec-

TABLE 3.—*Comparison of 0.5-inch with 1.0-inch plungers, on gelatin desserts*
(Swift & Company data)

20 G OF SAMPLE/100 ML OF WATER (ALL RESULTS IN TRIPLICATE)						
	0.5-INCH PLUNGER			1.0-INCH PLUNGER		
Tests Performed July 19, 1949:						
(a) Test formula	20	20	19	50	50	48
(b) 0.1 g gelatin added to (a)	20	20	20	56	58	58
(c) Dessert sample No. 1	20	19	19	46	46	48
(d) Dessert sample No. 2	16	16	16	30	32	30
(e) Dessert sample No. 3	24	24	26	68	84	72
Tests Performed July 20, 1949:						
(f) Test formula	20	20	21	48	49	48
(g) 0.1 g gelatin added to (f)	20	19	20	56	56	56
(h) Dessert sample No. 4	20	20	20	40	38	42
(i) Dessert sample No. 5	19	18	19	42	42	42
(j) Dessert sample No. 2	16	17	16	28	28	30
Tests Performed July 21, 1949:						
(k) Test formula	20	20	19	48	49	48
(l) 0.1 g gelatin added to (k)	21	20	20	58	58	56
(m) Dessert sample No. 6	25	24	24	70	72	70
(n) Dessert sample—orange	19	20	20	42	42	44
(o) Dessert sample—strawberry	20	19	20	36	35	38

ommendation of the 1.0″ plunger, for testing the jelly strength of gelatin dessert powders. The Swift data, which appears in Table 3, shows that the 1.0″ plunger is sensitive to the addition of 0.1 g of gelatin to a gelatin dessert test sample, while the same sample tested with the 0.5″ plunger cannot be distinguished from the basic test formula. Mr. Mehlenbacher's comments follow:

"It would appear from these data that there is more merit to the use of the 1-inch plunger than we first thought. It would be our suggestion that this sort of thing be repeated and rechecked by some other collaborators. If their findings agree with ours, it seems that it might be advisable to suggest adoption of the one-inch plunger"

It is recommended—

(3) That the method **9.12** (*Methods of Analysis*, A.O.A.C., sixth ed.) as rewritten and submitted to collaborative tests, be adopted as the official jelly strength method for gelatin dessert powders.

(4) That the A.O.A.C. adopt the specifications for the 1.0″ plunger as shown in Figure 2.

ACKNOWLEDGMENT

The Associate Referee wishes to thank Referee S. C. Rowe, of the Food and Drug Administration, for his valuable suggestions and helpful cooperation in recognizing the necessity for these revisions of the tentative

A.O.A.C. jelly strength methods. The full cooperation of all collaborators has been appreciated.

REPORT ON GELATIN DESSERT CONSTITUENTS

By JOSEPH H. COHEN (General Manager, Atlantic Gelatin Division, General Foods Corporation, Woburn, Mass.), *Associate Referee*

A study has been started on methods **9.13** and **9.15**, A.O.A.C., 6th Ed., for determining sucrose and dextrose in gelatin dessert powder. Collaborative work on the first phase of this study has been confined to the evaluation of the precision of the methods. No attempt has been made at this time to check the accuracy of the methods. For this purpose, it was not necessary to have samples of known composition. Hence, a number of packages of four different gelatin desserts were acquired and each was thoroughly mixed to obtain four master samples which were subdivided and sent in duplicate to each of the five collaborators.

RESULTS AND DISCUSSION

The results of the collaborative work are shown in Table 1. As indicated in the note at the foot of the table, the results of collaborator No. 5 are not included in the average and range figures because they are so obviously out of line. The Associate Referee has since been informed that the analyses were made by an inexperienced operator.

Variance analysis of these data by S. Harrison of the General Foods Central Research Laboratories indicates the following:

(1) Collaborators agree with each other sufficiently well so that the difference between samples is established to a very highly significant (1,000 to 1 odds or greater) degree.

(2) The difference between collaborators running sucrose is significant (odds 20 to 1 or greater) and for dextrose is so slight as to be insignificant.

(3) Systematic differences exist between collaborators determining sucrose. Taking Collaborator No. 1 as an arbitrary standard these errors may be expressed as:

Collaborator No. 2 = −0.6% sucrose
Collaborator No. 3 = −0.9% sucrose
Collaborator No. 4 = −2.6% sucrose

(4) No systematic differences exist between collaborators determining dextrose.

(5) Sucrose content of samples as determined in duplicate by different collaborators must differ from one another by more than 4.7% in order for that difference to be considered significant. Lesser differences can be attributed to the lack of reproducibility of the method and to personal factors connected with the collaborators.

TABLE 1.—*Summary of analytical results for sucrose and dextrose in gelatin dessert samples submitted for collaborative testing of A.O.A.C. methods*

COLLABORATOR NO.	SUCROSE—PER CENT				DEXTROSE—PER CENT			
	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D	SAMPLE A	SAMPLE B	SAMPLE C	SAMPLE D
1	82.7	69.4	77.9	84.5	−0.88	17.0	8.2	0.89
	82.7	70.0	78.5	83.6	1.267	16.7	8.5	0.51
2	83.33	71.52	78.79	80.91	−0.67	14.80	5.59	2.39
	81.82	72.12	77.58	80.61	0.73	15.05	7.17	2.27
3	81.92	68.79	77.27	82.58	0.608	18.77	9.54	2.05
	81.92	69.39	76.96	83.03	0.608	19.018	10.18	0.24
4	81.5	67.5	73.6	81.1	−0.9	17.1	11.4	−0.8
	82.3	67.6	73.6	81.3	−0.9	17.8	10.8	−1.0
5*	62.56	61.66	67.71	59.71	30.46	21.97	21.45	29.25
	55.48	59.52	67.74	60.48	34.41	20.88	21.21	27.47
Average	82.3	69.5	76.8	82.2	−0.1	17.0	8.9	0.8
Range	1.8	4.6	5.2	3.9	2.2	4.2	5.8	3.4

* Results of Collaborator No. 5 are so obviously anomalous that they are not included in the average or the range figures. The Associate Referee has since been informed that an inexperienced operator made the analyses.

(6) Dextrose content of samples as determined in duplicate by different collaborators must differ by more than 4.5% in order for that difference to be considered significant. Lesser differences can be attributed to the lack of reproducibility of the method and to personal factors connected with the collaborators.

COMMENTS OF COLLABORATORS

H. N. Ishler:

The samples were first run by the A.O.A.C. method following the instructions exactly. In every case, the addition of the HCl to the sample preliminary to the inversion caused the formation of a white crystalline precipitate which persisted throughout the entire inversion step and had to be removed by filtration before the invert samples could be read in the polarimeter. The readings obtained on these invert samples showed no consistency, duplicates did not even remotely compare, the results were neither reproducible nor reasonable.

Since this laboratory has consistently used sodium oxalate to delead sugar solutions, and since a consideration of solubility data and a quick examination of this precipitate showed it to be an oxalate with the approximate composition of potassium acid oxalate, a new series was set up using sodium oxalate as the lead precipitant. The results obtained by this method are those reported.

After the first precipitation, a clouding of the filtrate was noted and in some cases, the appearance of a precipitate when the samples were cooled to 20°C. preparatory to reading them in the polarimeter. This had to be filtered off before the

samples could be run. This could probably be avoided by cooling to 20°C. before filtration.

It also seems necessary for those laboratories using a polarimeter rather than a saccharimeter that some note be made in the procedure to indicate that the calculations of the Clerget formula are in sugar degrees, and polarimeter readings should be converted to the Ventzke scale before the results are calculated.

Edward F. Steagall:

It would seem to me that this method leaves much to be desired if any degree of accuracy is to be obtained. For one thing, there is no assurance that the final volume is exactly 200 ml. Another is that by using a half normal sugar solution instead of a normal sugar solution, the errors inherent in the method are multiplied two-fold when the polariscopic readings are converted into the readings of a normal sugar solution using the modified Clerget formula.

Accordingly, I would like to suggest that a study be made to determine if a normal sugar solution could be used. Also, I have wondered if the volume could be checked by measuring the volume in a volumetric flask after the adddition of the lead acetate but omitting any use of Filter-Cel until just previous to filtration.

CONCLUSIONS AND RECOMMENDATIONS*

On the basis of this first phase of the work, it is concluded that the reproducibility of the results is good. In fact, by comparison with most commonly used sugar methods, the reproducibility between collaborators is excellent.

It is recommended—

(1) That method **9.13** and **9.15**, A.O.A.C., 6th Ed., be continued as first action.

(2) That further work be carried out next year with samples of known composition in order to obtain a measure of accuracy as well as precision, bearing in mind the comments of the collaborators with regard to changes which may be made next year.

COLLABORATORS

H. N. Ishler, General Foods Central Research Laboratories, Hoboken, N. J.
Dorothy Montgomery, Food and Drug Administration, Federal Security Agency, Washington, D. C.
Edward F. Steagall, Food and Drug Administration, Federal Security Agency, Washington, D. C.
C. B. Streightif, United Chemical and Organic Products, Division of Wilson and Co., Inc., Chicago, Ill.
D. Tourtellotte, Charles B. Knox Gelatine Co., Inc., Camden, N. J.

ACKNOWLEDGMENTS

The Associate Referee wishes to acknowledge the assistance of Harold Rosenthal (Atlantic Gelatin Division, General Foods Corporation, Woburn, Mass.) in the coordination of this work and the preparation of this report; S. Harrison and H. N. Ishler (General Foods Central Research Laboratories, Hoboken, N. J.) for the statistical evaluation of analytical

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 52 (1950).

results; and the aforementioned collaborators who participated in this work.

REPORT ON GUMS IN FOODS

By F. LESLIE HART (Food and Drug Administration, Federal Security Agency, Los Angeles, California), *Referee*

Methods of Analysis, A.O.A.C., is scheduled for its 7th Edition in 1950. A review of methods for the determination of gums in foods already adopted by the Association is therefore indicated.

There are certain Selected References that, in the opinion of the Referee, should be listed at the ends of the respective chapters.

These references are:

22.143, after Title, add reference number, referring to *This Journal*, **28.601** (1945).

22.146, after Title, add reference number, referring to *This Journal*, **25.612** (1942).

22.147, after Title, add reference number, referring to *This Journal*, **24.575** (1941).

To Selected References, p. 344, add to reference 37, *This Journal*, **23.597** (1940); **28.245** (1945).

To Selected References, p. 555, add to reference 14, *This Journal*, **28.249** (1945); **32.524** (1949).

Progress reports were received from the Associate Referees on Gums in Cheeses and Gums in Cacao Products. The latter report was oral. Both had developed methods that were submitted to collaborative study, but the reports of collaborators showed that the methods needed further refinement before submission to the Association. No reports were received from other Associate Referees.

RECOMMENDATIONS*

It is recommended—

(1) That studies be continued on the detection of gums in soft curd cheeses and method **22.138–22.141** be adopted as first action, pending completion of these studies.

(2) That studies be initiated on the detection of gums in cheese foods and cheese spreads.

(3) That studies be continued on detection of gums in cacao products.

(4) That studies be continued on detection of gums in catsup and related tomato products.

(5) That method **33.57** be adopted as official.

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 53 (1950).

REPORT ON GUMS IN SOFT CURD CHEESE

By M. J. GNAGY (U. S. Food and Drug Administration, Federal Security Agency, Los Angeles, California), *Associate Referee*

The Associate Referee had intended to submit a report covering a revision of the present tentative method, **22.138,** in time for the 1950 revision of the *Book of Methods.* A considerable amount of work has been done, including much of a collaborative nature, and the results are very good. Some collaborators had trouble in that they secured blanks which were higher than normal. This fact made the Associate Referee feel that further collaborative work should be done before submitting the method to the Association.

It is recommended*—

(1) That further work be done upon the method for determining the presence of gums in soft curd cheeses.

(2) That the method be studied in regard to its application to the detection of algin (sodium alginate) in soft curd cheese.

(3) That the method be studied in its application to the determination of gums in cheese spreads and cheese foods.

No report was given on gums in cacao products.

REPORT ON MICROBIOLOGICAL METHODS

By GLENN G. SLOCUM (Food and Drug Administration, Federal Security Agency, Washington, D. C.), *Referee*

Your Referee had hoped to report substantial progress on the development of microbiological methods at this meeting. Real progress which would permit the designation of the several methods as "official" has not been achieved and, indeed, obstacles have arisen which require that two methods, **40.22–40.25,** and **40.26–40.27,** classed as tentative, be dropped.

One problem facing the Associate Referees arises from the difficulty in obtaining satisfactory collaborative data which will support a recommendation that a given method be made official. Bacteriological procedures generally do not give results reproducible to the same degree of precision inherent in chemical determination. Since we are dealing with living organisms, the preparation and distribution of satisfactory collaborative samples in itself constitutes a serious problem. It may be necessary, though undesirable, to confine collaborative work to local areas, where uniformity of samples can be maintained.

Standard bacteriological methods are being developed by other organizations, notably the American Public Health Association. It is, of course, desirable, if not imperative that A.O.A.C. methods be in essential

* For report of Subcommittee C and action of the Association, see *This Journal,* 33, 52 (1950).

agreement with other "standard" methods, or the control official will be faced with a difficult choice. A.O.A.C. methods for eggs and egg products, sugar, and nuts and nut products are similar to but vary from procedures for the same products as described in the latest edition of Standard Methods for the Examination of Dairy Products of the A.P.H.A. These methods must and will be coordinated.

A brief statement of the status of each fixed microbiological method follows:

Eggs and Egg Products.—The methods are generally satisfactory but require collaborative work and coordination of several minor points with A.P.H.A. methods. Method will remain as first action.

Sugar.—The report of the Associate Referee indicates present method is the best available. The collaborative data available are not sufficient to support a recommendation that the method be made official, and it will remain as first action.

Canned Vegetables.—Requires additional collaborative work. Method satisfactory and will remain first action.

Canned Fruits and Other Acid Canned Foods.—Method and, particularly, media not satisfactory in the hands of the Associate Referee and others. Since this method is "tentative" and further investigation will be required to develop a new procedure, it is recommended that the method be dropped.

Nuts and Nut Products.—It is recommended that this tentative method be dropped. Present method requires further study by the Associate Referee and coordination with the A.P.H.A. method.

Canned Meats; Canned Fish.—Methods for these products have been published in the *Journal* but have not achieved "tentative" status. They should be coordinated with methods for other non-acid foods (Canned Vegetables).

Frozen Fruits and Vegetables.—Methods generally satisfactory but require revision for clarity and collaborative work.

RECOMMENDATIONS*

(1) Referee concurs in the reports of the Associate Referees on Sugar and on Frozen Fruits and Vegetables.

It is recommended—

(2) That methods for Eggs and Egg Products, Sugar, and Canned Vegetables be continued as first action.

(3) That methods for Canned Fruits and Other Acid Canned Products, and for Nuts and Nut Products be dropped.

(4) That work be continued on Eggs and Egg Products, Sugar, Canned Vegetables, Canned Meat, Canned Fish, Acid Canned Foods, Nuts and Nut Products, and Frozen Fruits and Vegetables.

* For report of Subcommittee C and action of the Association see *This Journal*, 33, 54 (1950).

REPORT ON MICROBIOLOGICAL METHODS FOR FROZEN FRUITS AND VEGETABLES

By HARRY E. GORESLINE (Production and Marketing Administration, Poultry Branch, Washington, D. C.), *Associate Referee*

The report for 1948 consisted mainly of a set of suggested methods for the microbiological examination of frozen fruits and vetegables. The methods were published in the August *Journal*, pages 519 to 521. Reprints were obtained and forwarded to twenty-seven representative bacteriologists, who would have occasion to use such methods, with a request that their comments and criticisms be presented to your Referee. Replies were received from sixteen sources.

The comments received were favorable to the methods and no severe criticism or major changes were suggested. It was suggested by several that the use of *any* type of blender jar be allowed, because of breakage, instead of specifically stating "borosilicate glass mechanical blender jar." Suggestions were also made that equal time of incubation be given for both fruits and vegetables. Most of the suggestions for minor changes or corrections in the methods seem justified and should be applied in preparing a revision of the methods as published.

It is recommended* that the methods on microbiological methods for frozen fruits and vegetables be revised for greater clarity and usefulness and that collaborative work be undertaken, in which the application of the methods will be tested on prepared samples. It is recommended that the methods be continued in their present status until the revision and collaborative work has been completed.

REPORT ON MICROBIOLOGICAL METHODS FOR DETECTING AND ESTIMATING THERMOPHILIC BACTERIA IN SUGAR

By E. J. CAMERON (Research Laboratories, National Canners Association, Washington, D. C.), *Associate Referee*

Comparative tests were conducted this year on two culture media suggested as substitutes for the liver broth medium described in Sec. **40.14(b)** and the sulfite agar medium described in Sec. **40.14(c)** of the *Methods of Analysis*, A.O.A.C., Sixth Ed., 1945. The two media are used in detecting and estimating the numbers of thermophilic anaerobes in sugar.

The substitute medium for the liver broth consisted of tryptone 10 grams; dextrose 5 grams; yeast extract 1 gram; dibasic potassium phosphate 1.25 grams; and water 1,000 ml. The substitute medium for the sulfite agar simply replaced the iron strip or nail in the original agar

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 54 (1950).

with an organic source of iron in the form of ferric citrate (10 ml. of a 5% solution per 1,000 ml. of medium).

Six samples of sugar, representing varying degrees of contamination with sulfite spoilage organisms and thermophilic anaerobes not producing hydrogen sulfide, were sent to 23 different laboratories with experience in the bacteriological examination of sugar.

Although only 15 collaborators have reported so far, the trend of the results definitely indicates that the original liver broth and sulfite agar media are more sensitive than the substitute media tested.

In view of these preliminary findings, no changes in the present sugar methods are suggested.

REPORT ON ENZYMES

By J. WILLIAM COOK (U. S. Food and Drug Administration, Federal Security Agency, San Francisco, Calif.), *Referee*

The following recommendations are in accordance with the policy of the A.O.A.C. for the elimination of tentative methods. It is hoped that a constructive program on enzyme work may be started soon.

It is recommended*—

(1) That the method for proteolytic activity of papain be made first action. (Collaborative work was done and is reported in *This Journal*, **19**, 373 (1936).)

(2) That work be continued on proteolytic enzymes including papain.

REPORT ON OILS, FATS, AND WAXES

By J. FITELSON (Food and Drug Administration, Federal Security Agency, New York 14, N. Y.), *Referee*

The Associate Referee on unsaponifiable matter reports that further studies on the proposed modification of the official S.P.A. method disclosed several shortcomings. The Referee concurs in the recommendation that further studies in this field be discontinued. The Associate Referee on peanut oil has completed the work on the modified Bellier test and plans to review the quantitative methods for this oil. No report on antioxidants will be submitted, although methods for the detection of propyl gallate and similar compounds are now being investigated.

In preparation for the revision of the *Methods of Analysis*, the chapter on Oils, Fats, and Waxes has been carefully reviewed and several changes are proposed. Recommendations for minor revisions are made for methods, **31.1, 31.2, 31.3, 31.8, 31.18, 31.21, 31.22, 31.28, 31.32, 31.40, 31.41, 31.43,** and **31.49.** Many of these changes are editorial, while others involve

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 58 (1950).

slight modifications not affecting the fundamental procedures. These changes reflect current techniques and do not require collaborative studies.

The method for the determination of specific gravity at the temperature of boiling water (**31.6** and **31.7**) is no longer used, and its deletion as an obsolete method is recommended.

Several tentative methods have received collaborative study and their adoption as first action is recommended. The method for thiocyanogen number (**31.22** and **31.23**), gave acceptable results in this collaborative work.[1] The two methods for cholesterol and phytosterol (**31.35** and **31.36**), were studied and found to give correct and reliable results.[2] The digitonin procedure, **31.36**, is the more convenient method, but makes use of an expensive reagent and therefore both methods were adopted. These methods are widely used and it is recommended that both of them be retained. The method for foreign fats containing stearin in lard (**31.52**), has received considerable collaborative examination. A simpler version of the present method was adopted after such studies in 1920 and then modified after additional collaborative work.[3] The method was later revised to include additional calculations. In addition, more recent studies by British investigators has confirmed the reproducibility and value of this method.[4]

The tests for rosin oil (**31.44**), cold test (**31.49**), fish oil and marine animal oils in vegetable oils (**31.53**), and mineral oil (**31.54**) are essentially well established procedures and their transfer to this category is recommended.

The method for coal-tar colors (**31.56, 31.57**), is based upon standard techniques described in Chapter 21, Coloring Matters. Extensive experience with this method has demonstrated its value in the hands of experienced analysts. However, recent work by inexperienced chemists has shown some ambiguity in the instructions and the need for amplification of certain portions of the method. In view of its importance, it is recommended that this method be made first action, and that an Associate Referee be appointed to study the method in order to clarify and revise the procedure.

It is also recommended that official methods **31.58** to **31.70**, inclusive, be deleted. These methods were adopted in 1934 after collaborative studies. However, they originated in the U. S. Department of Agriculture and have been revised several times by that organization. It has been necessary for the Referee to depend upon these revisions to keep the A.O.A.C. methods up-to-date. Since these are the official methods of another organization, it is preferable for those interested in this field to obtain their information directly from the source.

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[1] *This Journal*, **21**, 443 (1938).
[2] *Ibid.*, **1**, 513 (1915).
[3] *Ibid.*, **3**, 432 (1920); **4**, 195 (1920).
[4] *Ibid.*, **19**, 97 (1930); *Analyst*, **65**, 623 (1940).

RECOMMENDATIONS*

It is recommended—

(1) That the changes in Chapter 31, *Methods of Analysis*, proposed by the Referee be adopted.

(2) That the changes in the S.P.A. method proposed by the Associate Referee on unsaponifiable matter be accepted and the method made official.

(3) That work on unsaponifiable matter be discontinued and this subject be dropped.

(4) That the changes in the modified Bellier test **(31.47, 31.48)** for peanut oil, proposed by the Associate Referee, be accepted and this method be adopted as official.

(5) That work on this modified Bellier test be discontinued.

(6) That studies on quantitative methods for peanut oil be initiated.

(7) That method for coal-tar colors in oils **(31.56)** be studied, with the view to clarifying and improving the procedure.

(8) That work on methods for the detection and estimation of antioxidants in oils be continued.

(9) That studies on spectroscopic methods for the analysis of oils be initiated.

REPORT ON PEANUT OIL

By G. KIRSTEN (New York District, Food and Drug Administration, Federal Security Agency), *Associate Referee*

No collaborative study was conducted during the past year, but application of the method to numerous samples of oil has confirmed the value and reliability of the modified Bellier test for peanut oil. Comments of some of the collaborators who participated in last year's study indicated that more specific directions for cooling were desriable.

It is therefore recommended*—

(1) That the sentence on page 508, *Methods of Analysis*, **31.48**, line 5, beginning "Cool soln. in H_2O . . . " be deleted and the following substituted:

"Insert thermometer and cool with continuous agitation so that the temp. falls at the rate of ca 1°C/min.," also that the sentence on line 7, beginning "It is essential . . . ", be deleted and the following substituted:

"Cooling may be accomplished in air if the temp. of the soln is higher than room temp. or by occasionally immersing in a water bath with a temp. of not more than 5°C below that of the soln. The flask should not be immersed below the level of the liquid in it, and agitation should be continuous, to prevent the premature formation of turbidity by local cooling. The soln may be agitated by stirring with the thermometer or by swirling the flask."

* For report of Subcommittee C and action of the Association, see *This Journal*, 33, 55 (1950).

(2) That the modified Bellier Test be made official.

(3) That further work on the Modified Bellier Test be discontinued.

(4) That studies on the quantitative determination of peanut oil be initiated.

REPORT ON UNSAPONIFIABLE MATTER

By G. KIRSTEN (Food and Drug Administration, Federal Security Agency New York, N. Y.), *Associate Referee*

Preliminary experiments in the use of a chromatographic method for the purification of unsaponifiable matter were reported at the 1948 meeting of the A.O.A.C. (1).

Further work confirmed the difficulties encountered in these experiments. Although some experiments indicated the probability of a satisfactory solution to these problems, the resultant method would be too lengthy to offer any advantages over the present procedure. In addition, Swain (2) reported that in the case of some oils there was incomplete elution of unsaponifiable matter from the alumina adsorption column by ethyl ether, thus giving low recoveries. For these reasons it was decided that further work on this method would be discontinued.

Swain (3) and Karnovsky, Rapson, and Black (4) showed that in the case of certain marine oils three extractions were not enough to effect complete removal of the unsaponifiable matter from the soap solution. Accordingly, it is proposed that the method be modified to provide for four extractions in the case of these oils.

It is also proposed that the wording in the method with respect to the washing of the ether extract be changed to remove any ambiguity that may be present.

It is recommended*—

(1) That the sentence "Make a total of four extractions in the case of marine oils, or other oils with a high unsaponifiable content," be added at the end of the first paragraph of **31.40**, p. 504.

(2) That the sentence in line 4, page 505, beginning, "Then wash alternately three times . . . ," be deleted and the following be substituted: "Then wash the ether solution three times with 20 ml portions of ca 0.5 N aqueous KOH, shaking vigorously each time, and follow each KOH washing with a 20 ml H_2O washing."

(3) That work on the determination of unsaponifiable matter be discontinued and the subject be closed.

REFERENCES

(1) *This Journal*, **32**, 367 (1949).
(2) SWAIN, L. A., *J. Fish Res. Bd. Can.* **7**, 389 (1948).
(3) ——— *Analyst*, **69**, 376 (1944).
(4) KARNOVSKY, M. L., RAPSON, W. S., and BLACK, M., *J. Soc. Chem. Ind.*, **66**, 95 (1947).

* For report of Subcommittee C and action of the Association, see *This Journal*, **33**, 55 (1950).

No report was given on antioxidants.

REPORT ON MEAT AND MEAT PRODUCTS

By R. M. MEHURIN (Meat Inspection Div., Bureau of Animal Industry, Washington 25, D. C.), *Referee*

No report has been received from the Associate Referee on soybean flour in sausages or from the Associate Referee on creatin and creatinin in meat extract, although some work has been done on these methods.

The Associate Referee on detection of horse meat in ground meat has proposed a chemical method based on the well known relatively high linolenic acid content of horse fat compared to the fat of other common food animals. Horse meat does not usually contain much fat and when moderate amounts are mixed with fat beef or pork, it is sometimes difficult to obtain positive results by use of the chemical method. The General Referee believes, therefore, that the precipitin method for the detection of horse meat should also be investigated. A prominent manufacturer of biological products states that they expect soon to place anti-horse serum on the market.

It is believed that the present tentative quantitative method for starch should be revised and subjected to collaborative study before being given a first action status.

Many old and seldom used tentative methods are recommended for deletion. Most of these have had no collaborative work. Several much needed methods are recommended for first action and are supported by collaborative or other experimental work. These methods are for the determination of added water in sausage, nitrites and nitrates, lactose, and a more rapid method for salt, a procedure for starchy flour in sausage, and a procedure for the detection of dried skim milk in meat products.

RECOMMENDATIONS*

It is recommended—

(1) That collaborative work be continued on methods for soybean flour in sausage, for creatin and creatinin in meat products, and for horse meat in ground meat.

(2) That an Associate Referee be again appointed for starch in meat products.

That the following changes be made in sec. **28.3** (tentative).

(a) Change " . . . for period of 1–2 hours." in **(a)** last line, to "½ hr."

(b) Change **(c)** to read as follows:

(c) *Added water.*—% of added water = % of water in the original mixture plus

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* For report of Subcommittee C and action of the Association, see *This Journal* 33, 53 (1950).

% water added, minus 4 times (% protein in the mixture less % protein in added substances, if any, such as dried skim milk, cereal, soybean flour, etc.).

$$\text{Added water} = \frac{W - 4P}{1 - .01W + .04P},$$

where W = % water found by analysis, P = % protein found by analysis ($N \times 6.25$) less % added protein, if any.

(3) Change status of above method to first action.

(4) Change reference (1) to *This Journal*, **12**, 407 (1929); *ibid*, **11**, 112 (1928). (Present reference refers only to action of Committee C.)

The above changes (in Sec. **28.3**) are recommended on following grounds:

(a) Unnecessary instruction; further, additional heating for 1 hour at 125°C. is excessive.

(b) Percentages at present are based on original sausage mixture plus added water; revised method of calculation bases percentages on original mixture only.

(c) This method is constantly used in control laboratories. The moisture determination is similar to official method under **27.8.** The nitrogen determinations are official. The ratio of 4 to 1 for the proportion of water to protein in sausage meat is approximately correct as shown in *This Journal*, **11**, 112 (1928), and in Referee's report on collaborative work on this method, *Ibid.*, **12**, 407 (1929).

(5) **28.1, 28.20,** and **28.42:** Designate as procedures.

(6) **28.9** and **28.10:** Delete.

(7) **28.11** through **28.16:** Make first action, after revisions.

(a) Change 4th and 5th lines from top of page 425 to read: " . . . H_2O, and determine nitrate N by comparing the reading of the color of a suitable aliquot with a standard curve."

(b) Delete last sentence of **28.16:** "Prepare . . . standing."

(c) Change status to first action. Published in *This Journal*, **18**, 459 (1935), and results of collaborative work published, *Ibid.* **22**, 597 (1939). The method determines both nitrate and nitrite nitrogen, the latter being determined as in **28.17** (below) and deducted.

Present reference (6) refers to committee action making method tentative—delete and replace with above 2 references.

(8) **28.17:** Change status to first action. This colorimetric method is widely used and is accurate, rapid, and the color is developed according to an official method. Determinations of nitrite in meat products were accurately made by this method in the collaborative work performed in connection with the determination of nitrate in **28.14.**

(9) **28.18:** Delete "Starch" from heading and substitute "Starchy flour." Starch is rarely used and the "starch" heading would not be appropriate for paragraph (*b*) recommended herewith. Also delete

" . . . starch or . . . " in 4th line. Also delete " . . . and quantitative determination should be made" since no quantitative method for flour is recommended at this time. Designate this paragraph as **(a)**. Add following procedure as **(b)**. (This additional procedure is a very useful screening method and is widely used for such purpose and as a semi-quantitative procedure; not applicable where cellulosic material is present other than that due to starchy flour and spice.)[1]

(10) **28.19**: Delete.

(11) **28.20**: Revise and make a procedure.

(12) **28.21** through **28.24**: Delete.

(13) **28.28** through **28.32**: Delete.

(14) **28.33**: First part of this official method being in sections **28.28** and **28.30**, both tentative and both recommended for deletion, makes it necessary to begin section **28.33** with all of **28.28** plus that part of **28.30** remaining after following deletions: "**28.28**" in line 1; "to avoid subsequent interference in the determination of creatin, **28.33**" in lines 3 and 4; "and reserve for determination—under **2.24, 2.25** or **2.26**" in lines 8 to 12, incl.; also delete following in **28.33**: "or remaining portion—in **28.31**," in 1st and 2nd lines.

(15) **28.34** through **28.36**: Revise and make first action.

(16) **28.37, 28.38,** and **28.47,** and **28.49** through **28.55**: Delete.

(17) **28.58** through **28.60**: Delete.

(18) **28.61**: Delete—official, but only a reference to chapter on preservatives and of little need.

(19) **28.62** and **28.63**: Delete.

(20) Revision of method for salt, **28.5** as first action, replacing present official method if necessary.[1]

This method is rapid, does not require ashing, is approximately same as the official method under **24.5–24.6,** and is widely used in preference to the present official method.

(21) That the method for detection of dried skim milk be made a procedure,[1] and a new method for determination of lactose be adopted as first action.[1] It is believed a method for lactose should be included in this chapter as this substance is frequently determined in sausage as a means of estimating the amount of dried skim milk present. Of the methods employing the copper reduction procedure after removal of monosaccharides with yeast, the one recommended appears most generally applicable and to have sufficient experimental work behind it to warrant first action.

[1] Details of the changes are incorporated in the revision of *Methods of Analysis*, 7th Ed. 1950.

REPORT ON HORSE MEAT IN GROUND MEAT

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

An attempt was made to organize a practical method for the determination of horse meat in ground meats such as hamburger, bologna, frankfurters, and sausage. It is a well established fact that horse fat contains considerable amounts of linolenic acid, while other common animal fats do not. Linolenic acid is precipitated as the hexabromide in cold ether solution. These facts are the basis for the present method.

Fisher (1) of the Connecticut Experiment Station used Paschke's (2) method for determining the hexabromide number of the extracted fat from 3 samples of horse meat and from beef, pork, and mutton. He reported figures of 86.1 to 127.4 mg hexabromide per gram of fat for horse fat, and only 2.8 to 9.2 for the other fats. Paschke (2) reported a hexabromide number of only 41.2 for horse fat and 2.8 to 3.3 for the other fats. Crowell (3), of the New Hampshire State Department of Health, modified the Paschke method and reported hexabromide numbers of 56.8 for horse fat, 2.0 for beef, and 7.5 for pork. Kass, Roy, and Burr (4) of the University of Minnesota, made a study of the determination of linolenic acid by the hexabromide method. They emphasize the empirical nature of the methods in use at present.

The Associate Referee has tried to simplify and standardize the procedure so that it can be performed easily. No attempt is made to determine the exact amount of linolenic acid present, but only to arrive at an empirical number that can be duplicated under controlled conditions. The method follows.

HEXABROMIDE METHOD

REAGENTS

(a) *Petroleum ether.*

(b) *Ethyl ether.*

(c) *Bromine.*

(d) *Alcoholic KOH.*—Dissolve 20 g KOH in 13 ml water and dilute to 100 ml with 95% alcohol.

(e) H_2SO_4 (1+4).

PREPARATION OF SAMPLE

Select portions containing maximum amount of fat and pass thru a meat grinder at least twice.

EXTRACTION OF FAT

Place enough of the prepared sample (50–100 g) to yield at least 2–3 g of fat in a 400 ml beaker, and place in air oven maintained at 90° for ca ½ hr. Remove, partially cool, and cautiously add portion-wise ca 100 ml of petroleum ether. The sample should be warm enough to cause the ether to boil vigorously, but not so violently as to be thrown from the beaker. Stir the mixture thoroly as it boils,

and let stand until ether layer clears. Carefully decant the clear layer to a clean 200-ml beaker, rinse the residue with ca 50 ml of the petroleum ether, allow to settle, and add this ether to the first portion. Evaporate just to dryness on a hot plate. If the ether fat soln is not perfectly clear it may be filtered thru a dry paper.

SAPONIFICATION OF FAT AND SEPARATION OF FATTY ACIDS

Weigh 1 g of the extracted fat (± 0.1 g) in a 50-ml beaker, add 2 ml of the alcoholic KOH soln, and boil for ca 3 min on a hot plate. Add 10 ml H_2O. Transfer the resulting soap soln to a 100 ml separatory funnel, using as small a portion of water as possible. Add 20 ml of ethyl ether and 2 ml of H_2SO_4 (1+4). Shake thoroly and allow to separate. Remove and discard the water layer. Transfer the ether layer to a 50-ml Erlenmeyer flask, using two 5–6 ml portions of ether to wash the funnel. Do not include any of the water layer. Evaporate this soln just to dryness on a hot plate, being careful not to heat too long after the ether has evaporated.

PRECIPITATION OF THE LINOLENIC HEXABROMIDE

Add 15 ml of dry ethyl ether to the resulting dry fatty acids and cool in a salt ice, alcohol, freezing mixture to –15°C. Slowly add 0.6 ml of Br with stirring. Allow to stand in the freezing mixture for 10–15 min. and then place in a refrigerator or other suitable place at not above 10° overnight.

While still cold, filter the resulting precipitate on a thin asbestos mat in a weighed Gooch crucible. Wash with three 15-ml portions of ethyl ether cooled to 0.5°. Dry in an oven for 30 min. at 100° and weigh. Calculate the results as mg/g of fat.

Preliminary tests give a hexabromide number of ca 100 on authentic horse fat and ca 2–4 on beef and pork.

It is recommended* that the study of the method be continued and applied to mixtures of horse meat and other meats, and known mixtures be sent to collaborators for final determination of the value of the method.

LITERATURE CITED

(1) FISHER, H. J., Conn. Agr. Exp. Station, Bul. 475 (1943).
(2) PASCHKE, BRUNO, *Z. Untersuch Lebensm.*, **76**, 476 (1938).
(3) CROWELL, G. K., *This Journal*, **27**, 448 (1944).
(4) KASS, J. P., ROY, W. R., and BURR, G. O., *Anal. Chem.*, **19**, 21 (1947).

REPORT ON NUTS AND NUT PRODUCTS

By A. M. HENRY (U. S. Food and Drug Administration, 416 Federal Annex, Atlanta 3, Ga.), *Referee*

The methods proposed and adopted as tentative last year were given a thorough trial in the examination of six samples of pecans by A. J. Shingler. The results are recorded in Table 1.

This work showed that the open Carius method is not accurate on nuts and its deletion is being recommended.

No offers of collaboration were received from the members of the A.O.A.C. An appeal to individual members was made and some collaborators were obtained. There has not been time for completion of the

* For report of Subcommittee C and action of the Association see *This Journal*, 33, 53 (1950).

TABLE 1.—*Examination of pecan samples*

	CURTIS	FROTSCHERS	MONEY MAKER	SEEDLINGS	SUCCESS	VAN DEMANS
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Moisture	2.53	2.97	2.82	3.05	2.73	2.36
Crude Fat						
Direct	71.30	70.25	—	72.07	71.71	76.60
Indirect	71.32	71.34	73.28	72.38	—	76.70
Crude Protein						
Fat not Extracted	9.62	10.28	8.61	10.28	—	8.90
Fat Extracted	9.15	9.89	8.35	10.32	10.66	8.74
Crude Fiber	2.13	2.02	2.00	2.03	1.93	1.93
Ash						
No Leaching	1.86	1.86	1.56	1.59	1.73	1.54
No Leaching	1.82	1.77	1.62	1.53	1.74	1.55
With Leaching	1.78	1.75	1.54	1.51	1.66	1.46
Reducing Sugar						
Before Inversion	0.09	0.11	0.18	0.05	0.09	0.07
After Inversion	6.13	4.77	4.55	4.48	3.77	3.81
Sucrose	5.57	4.30	4.03	4.08	3.40	3.44
Sodium Chloride	0.02	0.02	0.03	0.02	0.02	0.02
P_2O_5						
In Sample	0.65	0.74	0.66	0.68	0.80	0.67
In Ash	36.91	42.51	42.85	45.01	48.09	45.50
K_2O						
In Sample	0.12	0.12	0.10	0.09	0.10	0.09
In Ash	6.72	6.61	6.71	6.18	6.04	6.17

collaborative work. It is planned to complete this collaborative study of the methods in the coming year.

The minutes of the Meeting of International Commission on Fats and Oils in London, July, 1947, regarding the analysis of oleaginous seeds, which is applicable to many nut meats, were reviewed and found to be in accord with methods recommended last year.

RECOMMENDATIONS*

It is recommended—

(1) That methods for preparation and preservation of samples and microscopic examination under peanut butter be adopted as procedural methods.

* For report of Subcommittee C and action of the Association see *This Journal*, 33, 55 (1950).

(2) That all the above methods be given collaborative study in the coming year.

(3) That sorting methods for moisture and fat and methods for added starch in nut butters and pastes be studied.

(4) That the method for ash be amended to read "See **34.9** or **34.10** if added chlorides present." Under sodium chloride **(a)** Open Carius Method, and **(b)** be eliminated, and the present tentative methods be adopted, first action.

REPORT ON ECONOMIC POISONS

By J. J. T. GRAHAM (Production and Marketing Administration, Insecticide Division, Livestock Branch, Washington, D. C.), *Referee*

At last year's meeting of the Association of Official Agricultural Chemists an appeal was made for volunteers to act as Associate Referees, and the following chemists agreed to act in that capacity:

A. B. Heagy, Maryland Inspection and Regulatory Service, College Park, Maryland.
L. G. Keirstead, Agricultural Experiment Station, New Haven, Connecticut.
J. D. Patterson, State Department of Agriculture, Salem, Oregon.
J. B. La Clair, State Department of Agriculture, Sacramento, California.
Herbert A. Rooney, State Department of Agriculture, Sacramento, California.
C. V. Bowen, F. I. Edwards, E. E. Fleck, and S. A. Hall, Division of Insecticide Investigations, Bureau of Entomology and Plant Quarantine, U. S. Department of Agriculture, Beltsville, Maryland.

The Associate Referees have done a good job and their work is appreciated by the Association. Their reports speak for themselves and need no further comments or elaboration by the Referee.

No further report is as yet available on the study of methods for analysis of pyrethrum powder by members of this Association in collaboration with the Consultative Committee on Insecticide Materials of Vegetable Origin, of the Imperial Institute, London, of which mention was made in the Referee's report last year. The results of the collaborative work are being studied at the Imperial Institute and should be available for next year's meeting.

Last year the Referee mentioned a number of products for which methods of analyses might be studied if a sufficient number of chemists were interested and had the time available in which to take up the work. New subjects of study undertaken this year comprise organic thiocyanates, parathion, and the dimethyl dithio carbamates. Products for which methods have previously been studied and which are continued this year are rodenticides, herbicides, benzene hexachloride, DDT, tetraethyl pyrophosphates, and oil emulsions.

Other important economic poisons for which we have no official methods and which offer an interesting field for study are aerosol insecticides, products that contain chlordane, toxaphene, piperonyl butoxide, piperonyl cyclonene, zinc ethylene bis dithio carbamate, sabadilla alkaloids, coal-tar disinfectants, and the quaternary ammonium compounds.

New products are continually coming on the market, and recent introductions are the natural plant material, ryania, and synthetics, such as the chlorinated compounds "118" and "497." The Referee again, as at last year's meeting, requests volunteers to act as Associate Referees on economic poisons.

In connection with the preparation of the manuscript of the forthcoming seventh edition of the *Book of Methods*, following a request from the Chairman of the Editorial Committee, the Referee reviewed the chapter on Economic Poisons and has suggested a number of clarifying editorial changes, which have been submitted to that Committee.

The methods for determination of dichloro diphenyl trichloroethane, based on determination of total benzene-soluble chlorine, have been rewritten to conform with the general style of the book. No changes in the procedures have been made.

The procedure for determination of rotenone in derris and cubé powder, paragraph **6.110,** has been rewritten to conform to the style of the book, and has been slightly modified in the interest of obtaining more correct results. The method as rewritten conforms to the procedure that has been in use in the laboratories of the Insecticide Division for a number of years and has been found to be satisfactory. The use of a 50 ml flask for the precipitation instead of the 125 ml flask specified in the sixth edition is a decided advantage, because it is much easier to transfer all of the precipitate from the smaller flask to the crucible, and there is less chance of loss of the precipitate.

Since it has been decided that no tentative methods are to be included in the seventh edition, it was necessary to review the tentative methods in the sixth edition and to make recommendation for their proper disposition; accordingly, the following changes are recommended:

RECOMMENDATIONS*

(1) That the Alcohol Caustic Method for 2, 2-Bis (p-Chlorophenyl)-1, 1, 1-Trichloroethane (DDT), pars. **6.151–6.152,** be dropped.

(2) That Method III for determining total arsenic, pars. **6.9–6.10,** be adopted as first action.

(3) That the distillation method for determining total fluorine, pars. **6.22–6.23,** be adopted as first action.

(4) That the method for determining fluorine present as sodium fluosilicate, pars. **6.24.–6.25,** be adopted as first action.

* For report of Subcommittee A and action of the Association, see *This Journal*, 33, 39 (1950).

(5) That the method for determining total arsenic oxide, pars. **6.38–6.39,** be adopted as first action.

(6) That the official methods for the analysis of magnesium arsenate, pars. **6.50, 6.51, 6.52,** and **6.53,** be deleted.

(7) That the method for determining pyrethrin II in pyrethrum powder, par. **6.114,** be adopted as first action.

(8) That the method for determining pyrethrin I in pyrethrum extracts in mineral oil, pars. **6.115–6.116,** be adopted as first action.

(9) That the tentative iodine titration methods for the analysis of lime-sulfur solutions, pars. **6.123, 6.124, 6.127, 6.129** and **6.132,** be deleted.

(10) That the method for determining sulfide sulfur in lime-sulfur solutions, par. **6.130,** be adopted as first action.

The Referee concurs in the following recommendations of the Associate Referees:

(11) That Methods I through VI for determining DDT, as published in the Journal of the Association of Official Agricultural Chemists, **30,** 319 (1947) and amended, *ibid,* **31,** 368 (1948) be adopted as official methods.

(12) That the method for determining DDT in emulsions as published, *ibid,* **31,** 371 (1948) be adopted as official method.

(13) That Methods No. 20 and No. 21 for determining 2, 4-D in herbicides, as given in the report of the Associate Referee, be adopted as first action.

(14) That Methods No. 23A and No. 23B for determining 2, 4-D in herbicides be revised as suggested by the Associate Referee and subjected to further study.

(15) That the study of methods for determining parathion be continued.

(16) That the study of methods for determining oil in oil emulsions be continued.

(17) That the study of methods for the analysis of dimethyl dithio carbamates be continued.

(18) That the study of methods for determining organic thiocyanates in economic poisons be continued.

(19) That the method for determining alphanaphthylthiourea based on a determination of its nitrogen content as described in the report of the Associate Referee on rodenticides be adopted as first action, and that the method be given further study.

(20) That further work be done on methods for the detection and determination of sodium fluoroacetate (1080).

(21) That the method for determining tetraethyl pyrophosphate described in the report of the Associate Referee be adopted as first action.

(22) That the modified partition chromatographic method (Harris, T. H. "The Determination of Gamma Benzene Hexachloride in Insecti-

cide Products" *This Journal* **32**, 684 (1949), and the infrared spectrometer method (Tufts, L. E., and Kimball, R. H., "The Analysis of Hexachlorocyclohexane by Infrared Spectroscopy," Hooker Electrochemical Company Report No. 4706. May 15, 1949), for the determination of the gamma isomer in technical benzene hexachloride, be adopted as alternate methods, first action.

(23) That the modified partition chromatographic method for the determination of the gamma isomer of benzene hexachloride in wettable powder and insecticidal dust formulations be adopted as first action.

(24) That the investigation of methods for the determination of the gamma isomer of benzene hexachloride in emulsion concentrates, solutions, and formulations containing other organic insecticides be continued.

REPORT ON RODENTICIDES

By J. B. LaClair (Bureau of Chemistry, State of California Department of Agriculture, Sacremento 14, Calif.), *Associate Referee*

Though ANTU (alpha naphthyl thiourea) continues to be a popular and effective rodenticide very little work has been done toward a standardized method of analysis.

Elmore (*This Journal*, **31**, 366, 1948) reported that elemental analyses of ANTU showed that the nitrogen value gave the most nearly correct and consistent results.

A copy of the U.S.D.A., Insecticide Division, revised method 820.0 was submitted to one of the major manufacturers of ANTU for comment. The reply disclosed that their control method was quite similar, and that they approved the procedure.

A slightly revised procedure and samples were sent to collaborators for analysis.

INSTRUCTIONS, DESCRIPTION OF SAMPLES, AND PROCEDURES

Sample No. 1 is a technical grade alpha naphthyl thiourea, and is to be analyzed by method (1) Technical Alpha Naphthyl Thiourea.

Sample No. 2 contains approximately 5% of technical grade alpha naphthyl thiourea mixed with infusorial earth as a diluent. This sample is to be acetone extracted in a Soxhlet using procedure (2) Mixtures Containing Alpha Naphthyl Thiourea.

Sample No. 3 is a prepared bait containing approximately 2% of technical grade alpha naphthyl thiourea mixed with soya meal, ground wheat, fish meal, and bran mixed wtih a small amount of animal fat. This sample is to be treated first with petroleum ether to remove the grease and oils, and then extracted with acetone as under procedure (3) Alpha Naphthyl Thiourea Mixtures Containing Greasy Material.

All samples should be analyzed in triplicate.

Collaborative results are given in Table 1.

The details of the method are given in *Methods of Analysis*, 7th Ed., 1950.

COMMENTS OF COLLABORATORS A. B. HEAGY AND J. E. SCHUELER

(1) On the materials of the type represented by number 3, we believe it would be advantageous to modify the procedure as follows: Extract a weight of sample equivalent to approximately 0.2 gram of ANTU with petroleum ether in a Soxhlet apparatus for one hour. Withdraw petroleum ether and substitute acetone. Continue as directed. This saves manipulation time and avoids the possibility of losing any of the sample through incomplete transfer.

(2) Using sodium sulfide to remove the mercury caused a relatively high blank and some difficulty in securing a sharp end-point. For this reason we prefer to use the thiosulfate.

(3) It is suggested that 25 ml sulfuric acid be used in the digestion with a corresponding decrease in the amount of water added prior to distillation.

TABLE 1.—*Collaborative results on ANTU (alpha naphthyl thiourea)*

COLLABORATING CHEMIST	RESULTS IN PERCENTAGES OF ANTU FOUND		
	SAMPLE NO. 1	SAMPLE NO. 2	SAMPLE NO. 3
W. R. Flack, Eastern States Farmers' Exchange, Buffalo 5, New York	97.45	4.93	2.35
	97.45	5.01	2.26
	97.83	4.91	2.29
	Av. 97.58	4.95	2.30
J. A. Mezzapelle, Bureau of Chemistry, California State Department of Agriculture, Sacramento, California	98.63	4.92	2.34
	97.19	4.92	2.36
	100.79	4.96	—
	Av. 98.87	4.93	2.35
A. B. Heagy, Inspection and Regulatory Service, State of Maryland, College Park, Md.	98.29*	5.10*	2.44*
J. E. Schueler, Inspection and Regulatory Service, State of Maryland, College Park, Md.	98.28*	4.98*	2.35*
Total Averages	98.26	4.99	2.33
Wt. per cent of tech. ANTU added to sample		5.04	2.34
Per cent ANTU added, calculated to 98.26% ANTU in the tech. ANTU		4.95	2.30

* Average of six determinations.

DISCUSSION

The two prepared samples were purposely made difficult. Sample No. 2 contained a smaller percentage of ANTU than normally appears in commerce. Sample No. 3 was prepared from a number of materials which could appear in a commercial article.

The results as a whole were most gratifying. Very close agreement was obtained on the two prepared samples. The large factor used in converting nitrogen to ANTU causes some variation in the analysis of sample No. 1. (Technical ANTU).

The actual percentage of ANTU in the technical material used in this project is unknown, but the percentage from nitrogen seems logical and the calculated results at the end of the table show very good recovery for the procedures, and good agreement with theoretical calculations.

RECOMMENDATIONS*

It is recommended—

(1) That the method for determining ANTU in rodenticides from nitrogen be studied further, incorporating the collaborator's suggestion that petroleum ether extraction of greasy mixtures be made in a Soxhlet instead of by the procedure as written.

(2) That the determination of ANTU from nitrogen be adopted as first action.

(3) That further work be done to develop and test any promising method for the determination and/or detection of sodium fluoroacetate, "1080."

REPORT ON DDT

By Elmer E. Fleck (United States Department of Agriculture, Agricultural Research Administration Bureau of Entomology and Plant Quarantine, Beltsville, Maryland), *Associate Referee*

It is recommended* that methods (1) through (6) as published in *This Journal*, **30**, 64–66 (1947), and amended *ibid.*, **31**, 73 (1948), be adopted as official.

It is also recommended that the method for determining DDT in emulsions as published, *ibid.*, **31**, 72 (1948), be adopted as official.

That the Alcohol Caustic Method for 2, 2-Bis (p-Chlorophenyl)-1, 1, 1-Trichloroethane (DDT), pars. **6.151–6.152**, be dropped.

* For report of Subcommittee A and action of the Association, see *This Journal*, **33**, 39 (1950).

REPORT ON TETRAETHYL PYROPHOSPHATE

By S. A. HALL (U. S. Department of Agriculture, Agricultural Research Adminisration, Bureau of Entomology and Plant Quarantine Beltsville, Md.), *Associate Referee*

Analysis for tetraethyl pyrophosphate in insecticidal products is based upon the selective hydrolysis of the sample followed by a separation of the unhydrolyzed tetraethyl pyrophosphate from acidic ethyl phosphates and polyphosphates. Four methods (designated as M, V, H, and W) based upon this principle, but differing in technics of separation or hydrolysis, were described by your Associate Referee (1) at the 1948 A.O.A.C. annual meeting. Since that time the different methods have been subjected to critical comparison. It was found that the results obtained were comparable and satisfactory by any one of the four methods. Method V in its improved form, as recently published (2), was found definitely superior to the others from the standpoint of simplicity and speed of carrying out analyses. It was therefore selected for collaborative study. A slight modification of this method of Wreath and Zickefoose (2), which was found necessary to adapt it to commercial formulations of tetraethyl pyrophosphate, is described below.

METHOD

APPARATUS

(1) *Weighing buret* (5–10 ml. capacity).

(2) 100-*ml. buret* containing glass wool plug at bottom.

(3) 125-*ml. separatory funnel.*

(4) *Cylindrical funnel* approx. 1 inch diam. ×3 inches length packed with 1 inch of cotton.

(5) 250-*ml. volumetric flask,* 100-ml. pipette, and 50-ml. pipette.

(6) *pH meter (optional).*

REAGENTS

(1) *Acetone, 25% solution in water.*—500 ml of acetone (commercial grade), are mixed with 1500 ml. of water and cooled to 25°C.

(2) *Sodium hydroxide soln.,* 0.1 *N.*

(3) *Hydrochloric acid,* 0.1 *N.*

(4) *Indicator,* 0.1% aqueous soln of methyl red or chlorophenol red.

(5) Amberlite IR-4B resin, analytical grade.[1]

PREPARATION AND USE OF RESIN COLUMN

Screen the Amberlite resin to remove particles under 30 mesh. Weigh out 30 g of the screened resin, slurry with water, and pour into the 100-ml buret containing a small plug of glass wool at the bottom. Wash the resin column with 150 ml of 3% aqueous sodium hydroxide soln at a flow rate of ca 5 ml per min., and then rinse with water at a flow rate of about 25 ml per min. until the effluent is colorless to phenolphthalein. Wash with 25% acetone soln to displace the water. The column is then ready for use. Do not allow the column to run dry or channeling may result. Maintain the liquid level at all times ca 1 inch above the resin bed.

[1] Obtainable from Resinous Products and Chemical Co.; Philadelphia, Pa. or from Fisher Scientific Co., St. Louis, Mo.

(It is advisable to expand the resin bed after each determination before introducing a new sample, because the resin tends to pack in the column as it absorbs acidic material. This renovation is accomplished simply by back-washing with 25% aqueous acetone introduced by means of a large funnel and rubber hose at the base of the column until the liquid level reaches the top of the buret. After the resin settles, drain off to the customary height of 1 inch above the resin bed. The column is now ready to receive the next sample.)

(After eight to ten samples have been passed through the column, it is necessary to remove the absorbed acidic material. Regeneration of the resin is accomplished by repeating the initial treatment with 3% aqueous sodium hydroxide, water, and 25% acetone as described above).

(It is important, before introducing a sample, to test the effluent from the column. It should be colorless. If the effluent is yellow, wash the column with 25% aqueous acetone until the effluent is colorless.)

PROCEDURE

(I) *For purified or technical grades of tetraethyl pyrophosphate not mixed with a solvent, emulsifying agent, etc.*

Place the sample in the weighing buret and transfer a 2.5-g sample (1.0 g if the tetraethyl pyrophosphate content is over 50%), weighed to the nearest mg by difference, to 50 ml of 25% aqueous acetone contained in a 125-ml separatory funnel. Mix the sample with the acetone soln by swirling and allow to stand 15 min. at 25° ±2°C. Run the sample soln thru the resin column by gravity at a rate of ca 25 ml per min., catching the effluent in a 250-ml volumetric flask. Wash the funnel and column with three 50-ml portions of 25% aqueous acetone. Dilute the combined effluent to volume with water, mix, and transfer a 100 ml aliquot to a 250-ml beaker. Add 50 ml of 0.1 *N* sodium hydroxide, stir well, and allow to stand at room temp. for 30 min.; then back-titrate with 0.1 *N* hydrochloric acid to a *p*H of 6.0, using a *p*H meter. Methyl red or chlorophenol red may be used as an indicator if a *p*H meter is not available.

(II) *For formulations of tetraethyl pyrophosphate containing an organic solvent and emulsifying agent.*

Place the sample in the weighing buret and transfer a 2.5-g. sample, weighed to the nearest mg by difference, to 50 ml of 25% aqueous acetone contained in a 125 ml separatory funnel. Mix the sample with the acetone soln by swirling. If an oil tends to separate out, continue the swirling occasionally during 15 min. at 25 ±2°C. Run the sample soln thru a cylindrical funnel packed with 1 inch of cotton to absorb the oil and thence thru the resin column at a rate of ca 25 ml per min., catching the effluent in a 250-ml volumetric flask. Wash thru the separatory funnel, cylindrical funnel, containing the cotton, and the resin column with three 50-ml portions of 25% aqueous acetone. The procedure from this point is the same as in (I).

Calculate the percentage of tetraethyl phrophosphate as follows:

$$\frac{\text{Net ml. of 0.1 } N \text{ NaOH} \times 3.67}{\text{Weight of sample*}} = \% \text{ Tetraethyl pyrophosphate.}$$

COLLABORATIVE SAMPLES

Sample A—High grade tetraethyl pyrophosphate of about 90% purity.
Sample B—A typical technical grade of tetraethyl pyrophosphate.

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* NOTE: Original weight taken, not an aliquot weight based on 100 ml/250 ml.

Sample C—A typical formulated tetraethyl pyrophosphate insecticide concentrate containing an organic solvent and emulsifying agent.

TABLE 1.—*Collaborative results*
Per cent tetraethyl pyrophosphate

ANALYST	SAMPLE A	SAMPLE B	SAMPLE C	NOTES
Gordon	86.9	35.6	22.7	(1)
	88.5	36.3	22.8	(2)
	91.3	36.6	22.6	
	87.4			
	87.4			
	88.0			
	88.4, 88.8			(3)
Heagy	86.0	35.5	23.3	(1)
	86.5	36.2	23.4	
	86.5	35.1	23.2	(4)
	86.3	35.5	23.5	
	86.1			
Hall	89.2	35.8	23.6	(4)
	89.2	36.3	23.5	
	88.9	36.6	23.5	
Wreath	89.0	36.7	23.2	(4)
	90.0	36.9	23.0	(5)
	89.0	36.4	23.4	
Ave.	88.0	36.1	23.2	

NOTES: (1) Sample A, purified tetraethyl pyrophosphate is very hygroscopic; hence it decreases in assay value when transferring and weighing in a warm humid atmosphere such as prevailed in Washington, D.C., during the summer when these analyses were made.
(2) Gordon used chlorophenol red as an indicator and preferred it to methyl red in sharpness of end point.
(3) On sample A, Gordon obtained 88.4% using chlorophenol red and 88.8% on an aliquot using the *p*H meter to *p*H 6.
(4) Heagy, Hall, and Wreath used a *p*H meter to *p*H 6.
(5) Wreath also ran a sample of highly purified distilled tetraethyl pyrophosphate and obtained a result of 98.0%.

RECOMMENDATION*

It is recommended that the method as described be adopted, first action.

REFERENCES

(1) HALL, S. A., *This Journal*, **32**, 377–383 (1949).
(2) WREATH, A. R., and ZICKEFOOSE, E. J., *Anal. Chem.*, **21**, 808–810 (1949).

* For report of Subcommittee A and action of the Association see *This Journal*, **33**, 39 (1950).

REPORT ON 2, 4-D HERBICIDES

By A. B. HEAGY (Maryland Inspection and Regulatory Service, College Park, Md.) *Associate Referee*

HISTORY

The collaborative study of macro methods devised by the Bureau of Chemistry, Sacramento, California, was first undertaken during the year of 1948 at the recommendation of the Association.* As a result of this work the Associate Referee suggested that tentative methods No. 20 and No. 21 be subjected to another year's study with emphasis being placed on certain indicators with the purpose of improving and standardizing the end point.

In the case of tentative method No. 23, it was determined that the isopropyl ester formed emulsions throughout the procedure. Results from some 21 laboratories showed a high of 51% and a low of 1.50%. In view of these difficulties it was recommended that this procedure be studied further.

Also, it was proposed that a method (No. 23B), developed by the Dow Chemical Company, be examined in conjunction with the work on ester type materials.

Current collaborative work entailed the examination of 2 samples by methods No. 20, No. 23A, and No. 23B. Sample No. 1 was a 60% 2,4-D acid material purchased from the dealer's shelf, and No. 2 was a methyl ester of 2, 4-D supplied by the Thompson Hayward Chemical Company.

Five industry and 13 State laboratories cooperated. Most of those offering to take part in this work completed the study; however, several did not examine the ester product by tentative method No. 23A.

Tables of results have been distributed for convenience in comparing the indicators, *p*H meter, titrimeter, and various other methods used.

DISCUSSION

Results on sample No. 1 show good agreement among the phenolphthalein, thymolphthalein, and the *p*H meter. Thymolphthalein gave slightly higher results than the phenolphthalein or *p*H meter. The Associate Referee failed to suggest that both indicators be used in the standardization of the sodium hydroxide. If this had been done the spread would have been considerably lessened. This fact has been noted in the method submitted to the Committee on Methods Revision. Other changes and additions have been included: Filtering the ether solution through a plug of cotton; also, evaporating the final 25 ml. of the ether at room temperature by means of a current of air.

Comments from collaborators show a division of opinion as to the merits of the two indicators. Phenolphthalein is principally preferred

* Heagy, A. B., *This Journal*, 32, 383 (1949).

TABLE 1.—*Sample No. 1, tentative method No. 20*

COLLABORATOR	PHENOL PETHALEIN	THYMOL PETHALEIN	pH METER (8.5)	OTHER METHODS
	State Control Laboratories			
A	59.54	59.80	59.64	
B	59.94	60.06		60.51*
C	59.30	59.70		
D	58.70	60.30		
E	59.97	59.92		
F	59.69	60.00		
G	60.32		60.14	61.12†
H	58.77	59.20		60.53*
I	59.78	59.89	59.56	
J	59.17	59.22	58.84	
K	59.02	58.97	58.72	
L	59.68	60.32	59.62	59.38‡
M	59.80	60.33	59.71	59.39‡
Ave.	59.51	59.81	59.46	
	Industry Laboratories			
A	59.46	59.49	59.50	
B	59.38	60.11		
C	59.86	60.21		
D	59.68	60.16	58.89	
E	59.86	60.34	59.49	
Ave.	59.65	60.06	59.30	
General Ave.	59.55	59.88	59.41	

* Parr Bomb Method.
† Bromthymol Blue Indicator.
‡ Titrimeter *p*H 8.5.

because of the sharper end point. In some cases chemists reported check results using both indicators and feel either can be used successfully. It is the concensus of opinion that the *p*H meter should be rated first, followed by phenolphthalein and thymolphthalein.

Sample No. 2 by the two methods under consideration gave such erratic results that no attempt was made to arrive at any definite conclusions. The methyl ester material did not form emulsions, but judging from results obtained, some of the steps in the procedure need further clarification. Certain suggestions, given below, were offered by collaborators, and possibly will improve these methods.

(1) After addition of the $BaCl_2$, it has been recommended that a definite time of standing elapse before filtering to allow complete precipitation of fatty acid.

(2) Addition of a definite excess, for example, 10 ml of 1–1 HCl in part 3 before extracting with ether.

(3) In part 2, use of the same indicator in adjusting *p*H which will be used in the titration.

TABLE 2.—*Sample No. 2. Tentative methods No. 23A & No. 23B*

COLLABORATOR	METHOD 23A	METHOD 23B	OTHER METHODS
	State Control Laboratories		
A	40.86		
B	40.82	41.94	42.03*
C		45.55	
D	40.10	46.70	
E	39.76		
F	42.26	43.64†	
G	41.31	39.76	
H	39.48	41.32	
I		38.47	
J		38.22	
K	39.58	40.08	
L	39.41	40.15	
	Industry Laboratories		
A	41.11	41.01	
B	37.69	39.09	
C	43.68	41.75	
D	40.28	42.22	39.74‡
E	39.69		40.92§

* Combustion Furnace.
† Phenolphthalein Indicator.
‡ T-H Direct Titration.
§ Sodium Fusion Method.

(4) In Method 24B, one collaborator pointed out that a blank determination of free acid was very difficult to make, because of saponification of the methyl ester present. It might be well to direct attention toward overcoming this difficulty in subsequent studies.

(5) It was recommended that a blank titration of the alcohol be used, in preference to neutralized alcohol. By this means it is possible that any rearrangement of the indicator to an acid form is prevented, and the corresponding error eliminated.

(6) Use of isopropyl alcohol instead of ethyl alcohol has been suggested for next year's study.

RECOMMENDATIONS*

It is recommended—

(1) That methods No. 20 and No. 21 be adopted as first action.

(2) That methods No. 23A and No. 23B be revised as suggested and subjected to further study.

LIST OF COLLABORATORS

Industry:

W. R. Flach, Laboratory Director, Eastern States Farmers' Exchange.
R. W. Towne, Development Department, Monsanto Chemical Company.
H. A. Thomson, Control Chemist, Naugatuck Chemicals.
J. W. Zabor, Director of Development, Pittsburgh Coke & Chem. Co.
L. S. DeAtley, Laboratory Director, Thompson Hayward Chemical Co.

* For report of Subcommittee A and action of the Association, see *This Journal*, 33, 39 (1950).

State Officials:

Clemens Olsen, Arizona, Inspection Laboratory.
Herbert A. Rooney, California, Bureau of Chem., Dept. of Agriculture.
Charles W. Marshall, Canada, Dominion Dept. of Agriculture.
E. R. Winterle, Florida, Chemical Div., Agriculture Department.
A. C. Keith, Kansas, Control Div., Board of Agriculture.
John E. Schueler, Maryland, Inspection & Regulatory Service.
Albert B. Heagy, Maryland, Inspection & Regulatory Service.
Stacy B. Randle, New Jersey, Agricultural Experiment Station.
Howard C. Hammond, North Dakota, State Laboratories Department.
Miss Arlene Muffat, South Dakota, Chemical Laboratory.
Boyd L. Samuel, Virginia, Dept. of Agriculture & Immigration.
Miss Edith L. Lawrence, Washington, Agriculture Experiment Station.
Mrs. Edith E. Huey, Washington, Agriculture Experiment Station.

METHOD NO. 20 FOR THE DETERMINATION OF 2,4-DICHLOROPHENOXYACETIC ACID

(1) Transfer a sample equivalent to 1 g of 2,4-D acid to a 250-ml beaker, add 25 ml. of 1 *N* NaOH, and 50 ml of water. Warm for 15 min. and adjust to room temp. Stir to dissolve the 2,4-D. Filter and wash any insoluble matter that may be present and transfer to a 250 ml separatory funnel, neutralize with 10% H_2SO_4, and add 10 ml in excess.

(2) Extract the aqueous phase twice with 75-ml portions of ether. Wash the combined ether extracts free from mineral acid with 3, 10-ml portions of water.

(3) Filter the ether soln thru a funnel containing a small piece of cotton previously saturated with ether into a 400-ml beaker, rinsing the separatory funnel with ether. Add 25 ml of water, a few boiling chips, and evaporate off the ether layer on a steam bath until ca 25 ml of ether remains. Remove the beaker from the steam bath and evaporate off the remaining portion of ether at room temp. by means of a current of air.

(4) Dissolve the aqueous mixture in 100 ml of neutral ethyl alcohol and titrate with 0.1 *N* NaOH using 1 ml of indicator[1] (1 g. in 100 ml. of alcohol).

(5) Each ml of 0.1 *N* NaOH is equivalent to 0.0221 g of 2,4-Dichlorophenoxyacetic acid.

METHOD NO. 21 FOR THE DETERMINATION OF SALTS OF 2,4-DICHLOROPHENOXYACETIC ACID

(1) Dissolve a sample equivalent to ca 1 g of 2,4-D acid in 50 ml of water (filter and wash samples containing insoluble carriers), transfer to a 250-ml separatory funnel. Proceed as directed in Method No. 20, par. 1, beginning "Neutralize with 10% sulfuric acid."

NO. 23—A: DETERMINATION OF ESTERS OF 2,4-DICHLOROPHENOXYACETIC ACID IN PRESENCE OF SOAP, ACIDS, ALCOHOLS AND OILS

(1) Reflux a sample of weight equivalent to 0.7 g of the ester with ca 1 g of KOH and 90 ml of 95% ethyl alcohol for one hour in a 250 ml S/T Erlenmeyer flask. Transfer the alcoholic soln after saponification and cooling to a 250-ml separatory funnel with 80 ml of water and extract with 75 ml of petroleum ether. Draw off the alcohol-water phase into another 250-ml separatory funnel, extract with 75 ml petroleum ether. Draw off alcohol-water phase, wash ether layer a few times with 10 ml. portions of water, add washings to alcohol-water soln and evaporate down to

[1] Either phenolphthalein or thymolphthalein may be used in the titration provided the one selected is used in the standardization of the sodium hydroxide.

NOTE: If the sample does not contain an insoluble carrier, transfer 1 g of the 2,4-D acid into a 250-ml Erlenmeyer flask. Dissolve in 75 ml neutral ethyl alcohol and titrate with 0.1 *N* NaOH.

ca 50 ml on a steam bath. Make residue to ca 100 ml with water, cool, and transfer to a 200-ml volumetric flask.

(2) Add a few drops of 1% phenolphthalein solution and a few drops of a 1 to 1 solution of hydrochloric acid to the disappearance of the pink color, and then add 1 to 1 ammonium hydroxide solution until slightly alkaline. Add sufficient water to give a volume of about 150 ml. Add slowly sufficient 10% barium chloride solution to precipitate the fatty acids, make to volume, shake, and filter. The solution must be alkaline after the addition of barium chloride; otherwise the 2,4-D will precipitate.

(3) Transfer a 100-ml aliquot in a 250-ml separatory funnel, and acidify with hydrochloric acid. Extract the aqueous phase twice with 74-ml portions of ether. Wash the combined ether extracts free from mineral acid with 10-ml portions of water (three washings will be adequate).

(4) Transfer the ether soln to a 400 ml beaker, rinsing the separatory funnel with ether. Add 25 ml of water, a few boiling chips, and evaporate the ether layer on a steam bath.

(5) Dissolve the aqueous mixture in 100 ml of neutral ethyl alcohol and titrate with 0.1 *N* sodium hydroxide, using thymolphthalein as an indicator.

(6) Each ml of 0.1 *N* sodium hydroxide is equivalent to .0235 g of Methyl 2,4-Dichlorophenoxyacetate.

NO. 23 B: DETERMINATION OF ESTERS OF 2,4-DICHLOROPHENOXYACETIC ACID IN PRESENCE OF SOAP, ACIDS, ALCOHOLS AND OILS

REAGENTS

2. **(a)** Standard KOH-diethylene glycol[1] soln. Dissolve 12–13 g of KOH in 100 ml of diethylene glycol by warming. The soln will turn brown in color, but this will not affect the titration. Standardize the soln by weighing 2–3 g accurately in a 125-ml. Erlenmeyer flask, dilute with 25 ml of water, and titrate with 0.1 *N* HCl, using phenolphthalein indicator. (Do not warm. Note (a).) One g of the standard soln is equivalent to about 15 ml of 0.1 *N* HCl. For best results this factor should not be less than 13 or more than 17 ml 0.1 *N*/gram.

(b) Hydrochloric acid, standard 0.1 *N*.

(c) Sodium hydroxide, standard 0.1 *N* soln.

(d) Phenolphthalein indicator, 0.1% in a 1–1 mixture of ethanol and water.

(e) Bromthymol blue indicator, 0.04% in water.

PROCEDURE

3. **(a)** Weigh ca 3.000 g of standard KOH-diethylene glycol soln in a dry 125-ml Erlenmeyer flask. Calculate the theoretical weight of the product that will be saponified by this weight of soln, assuming 45% ester, and weigh into the flask about 0.2–0.3 g less than this calculated amount of sample. This will give ca 5-ml 0.1 *N* back-titration.

(b) Heat this mixture, with swirling, on a hot plate until it just starts to boil (first bubbles appear). Keep at this temp. for ca one min. Allow to cool for ca 5 min., then add 15–20 ml of water, and warm. The soln may have a reddish color which will obscure the end point. However, this color can usually be destroyed by boiling the soln at this point. If the soln is still too colored it may be titrated potentiometrically, the mid-point of the break being at a *p*H of 8–8.5.

(c) Cool to room temp. add ca 0.5 ml of phenolphthalein soln, and titrate with 0.1 *N* HCl until further addition causes no color change. The end point is more easily seen if the volume of soln is kept small.

(d) The small amount of free acid present in the sample is determined by adding

[1] Carbitol or ethylene glycol may be substituted.

10.0 g to an alcohol-water soln and titrating with 0.1 *N* sodium hydroxide potentiometrically, or to a bromthymol blue end point (*p*H 8). (Note 3) This acid is calculated as dichlorophenoxy-acetic acid.

CALCULATIONS

4. **(a)** The per cent ester is calculated as follows:

$$\% \text{ Ester} \div \text{acid calc. as ester} = \frac{\text{net ml. 0.1 } N \text{ KOH} \times 0.0263}{\text{sample weight}} \times 100$$

$$\% \text{ Free Acid as 2,4-D} = \frac{\text{ml 0.1 } N \text{ NaOH} \times 0.0221}{\text{sample weight (10.0 g)}} \times 100$$

$$\text{Acid correction} = \% \text{ Acid} \times 1.19.$$

$$\% \text{ Ester} = (\% \text{ ester} \div \text{acid calcd. as ester}) - (\text{acid correction})$$

(b) Example:

1.000 grams KOH-diethylene glycol soln. = 15.23 ml 0.1 *N* HCl

	(1)	(2)
Weigh KOH solution	5.265 g	3.466 g
Weigh sample	4.599 g	3.012 g
Wt. KOH soln as 0.1 *N* KOH	80.18 ml	52.79 ml
Back titration 0.1 *N* HCl	1.10 ml	0.90 ml
Net ml 0.1 *N* KOH	79.08	51.89
% Ester ÷ acid calc. as ester	45.23	45.32

$$\% \text{ Free acid as 2,4-D} = \frac{1.67 \times 0.0221}{10.0} \times 100 = 0.59$$

$$\text{Acid correction} = 0.59 \times 1.19 = 0.70$$

% Ester: (1) $45.23 - 0.70 = 44.53$

(2) $45.32 - 0.70 = 44.62$

Average 44.58

NOTES

5. **(a)** The standard KOH-diethylene glycol will change normality appreciably when heated alone. With the short heating period used in this determination it is impossible to duplicate conditions with a blank. Therefore, it is better to use only a small amount of excess KOH so that this error is not appreciable. With a 10-ml back-titration the error amounts to 0.05 ml or less. The back-titration may be considerably lower than this. With a sample of known concentration, good results were obtained with a titration of about 1 ml 0.1 *N* HCl.

(b) Saponification. The heating period required for complete saponification is very short. The temperature required is 140–150°C. for a minute or less. However, a thermometer is not necessary, as the first appearance of condensate on the side of the flask indicates the proper temperature. In the case of samples of 94–100% ester the solid potassium salt appears on first heating or upon standing at room temperature for 10–20 minutes. It is necessary only to melt this solid to complete saponification.

(c) Phenolphthalein cannot be used as an indicator in the titration of free acid, as the ester begins to saponify at the higher *p*H of this end point and high results are obtained.

REPORT ON OIL EMULSIONS

By L. G. KEIRSTEAD (Agricultural Experiment Station, New Haven, Conn.), *Associate Referee*

The work this year continued the 1948 program.[1] Four powdered materials were tried as adsorptive agents. Petroleum ether was compared with a mixture of this solvent with benzene (4+1) as eluants. Adsorptive agents tested were the following:

Absorptive powdered magnesia No. 2642. (Westvaco Chlorine Products Co., Newark, California.)
Acid silicic analytical reagent, precipitated powder. (Mallinckrodt Chemical Works, St. Louis, Mo.)
Alumina, adsorption, Fisher, 80–200 mesh. (Eimer and Amend, New York, N. Y.)
Attapulgus clay, 30–60 mesh. (Attapulgus Clay Co., Philadelphia, Pa.) (This reagent was ignited at 482° in a muffle for one hour before use.)
Hyflo Super Cel. (Eimer and Amend, New York, N. Y.)

The chromatographic tubes used were the No. J-1661, Size II, tubes of the Scientific Glass Apparatus Co., Bloomfield, N. J. These tubes have a diameter of 19 mm. and are 200 mm. long. A "Fisher Filtrator" was used for drawing the solvents through the columns of adsorbent.

Subsequent to last year's work it was noted that others had applied the principle of chromatographic adsorption to the determination of mineral oils. Both Koch[2] and Brooks, Peters, and Lykken[3] used Attapulgus clay as the adsorptive agent; because of their experience this reagent was added to the list of materials examined by this laboratory.

The following method was employed with all absorptive agents and both solvents:

METHOD FOR DETERMINING OIL BY CHROMATOGRAPHIC ADSORPTION

Place a wad of cotton in the bottom of the tube, apply suction and add the adsorptive agent in portions, tamping after each addition, until the height of adsorbent in the tube is 7 cm. On top of the column of adsorbent place a 1-cm layer of anhydrous Na_2SO_4. Saturate column with solvent, leaving 2 or 3 cm of solvent above the Na_2SO_4 layer. Weigh (by difference) about 2 g of oil or emulsion into the tube and wash thru the column into a 150-ml beaker with three 25-ml portions of solvent, using suction. Place the beaker on top of an oven set at 100°, let stand overnight, heat further as indicated, cool in a desiccator, and weigh.

The influence of the solvent on the recovery of oil was studied first. Four commercial mineral oils were treated as directed above, using a 1:1 mixture of absorptive powdered magnesia No. 2642 and Hyflo Super-Cel as the adsorbent; and petroleum ether alone, and a 1+4 mixture of benzene and petroleum ether, as the eluants. The recovered oil was heated 1 hour on a water bath and 20 min. in an oven at 100° before weighing.

[1] *This Journal*, **32**, 393 (1949).
[2] *Ind. Eng. Chem., Analyt. Ed.*, **16**, 25 (1944).
[3] *Ibid.*, **18**, 544 (1946).

Results are shown in Table 1:

TABLE 1.—*Influence of solvent on recovery of undiluted oils*

OIL	OIL RECOVERED	
	PETROLEUM ETHER AS SOLVENT	BENZENE+PETROLEUM ETHER (1+4) AS SOLVENT
	per cent	*per cent*
A	93.24	94.72
B	94.37	97.28
C	93.24	97.09
D	97.86	98.22
Average	94.68	96.83

The benzene-petroleum ether mixture obviously gave a better recovery of oil than did petroleum ether alone. Other adsorptive agents were then tried, using benzene-petroleum ether (1+4) as the only solvent, but drying the recovered oil by two methods (one hour on the water bath alone and one hour on the water bath plus 20 minutes at 100° in an oven; both after standing overnight on top of the oven, as noted in the "Method"). Results are given in Table 2:

TABLE 2.—*Influence of adsorbent and method of drying on recovery of undiluted oils*

OIL	Adsorbent							
	SILICIC ACID		MgO+SUPER CEL, 1+1		ALUMINA		ATTAPULGUS CLAY	
	WATER BATH	WATER BATH +OVEN	WATER BATH	WATER BATH +OVEN	WATER BATH	WATER BATH +OVEN	WATER BATH	WATER BATH +OVEN
	% recovery	*% recovery*	*% recovery*	*% recovery*	*% recovery*	*% recovery*	*% recovery*	*% recovery*
A	97.51	97.01	95.10	94.72	95.54	95.31	89.92	89.67
B	98.09	97.84	97.48	97.28			96.26	96.01
C	98.26	98.16	97.17	97.09			95.53	95.23
D	99.10	99.01	98.27	98.22			98.39	98.24
Ave.	98.24	98.01	97.01	96.83			95.03	94.79

Results in Table 2 (as in Table 1) are averages of triplicate determinations in each case. Table 2 shows clearly that silicic acid gives the best recovery of oil, averaging 98.2 per cent for four distinct types of commercial oil, and also shows that heating on the water bath for one hour, after most of the solvent has evaporated at a low temperature, is sufficient to remove the solvent.

Three commercial so-called "miscible oils" of unknown oil content were treated by the method, using three different adsorptive agents, and benzene-petroleum ether (1+4) as the solvent in each case, drying the recovered oils one hour on the water bath before weighing. Results (averages of triplicate determinations) are given in Table 3.

TABLE 3.—*Recovery of oil from commercial emulsifiable mixes ("miscible oils")*

	Absorbent		
MISCIBLE OIL	SILICIC ACID	MgO+SUPER CEL, 1+1	ATTAPULGUS CLAY
	% oil found	*% oil found*	*% oil found*
X	98.96	98.27	97.30
Y	86.32	86.13	83.76
Z	81.93	82.63	80.87

All three adsorbents removed the emulsifiers from all of the mixes to practical completeness, as indicated by the fact that the recovered oils did not emulsify on vigorous shaking with water, with one exception. The oil recovered on passing mix Z through the MgO-Super Cel mixture still emulsified somewhat with water.

Silicic acid proved to be the best adsorbent for separating these mixes,

TABLE 4.—*Analyses of mineral oil emulsions containing known amounts of oil*

	OIL FOUND, PER CENT	RECOVERY, PER CENT	OIL FOUND, CORRECTED FOR 1.91 PER CENT OF OIL B NOT PASSING COLUMN, PER CENT	RECOVERY, PER CENT
		Sample 1—97.00 per cent Oil B		
	95.42	98.4	97.28	100.3
	94.87	97.8	96.72	99.7
	95.50	98.5	97.36	100.4
	95.01	98.0	96.86	99.9
	95.68	98.6	97.54	100.6
	94.97	97.9	96.82	99.8
	94.46	97.4	96.30	99.3
	93.12	96.0	94.93	97.9
	94.74	97.6	96.58	99.6
	93.92	96.8	95.75	98.7
Ave.	94.77	97.7	96.61	99.6
		Sample 2—83.00 per cent Oil B		
	75.91	91.5	77.39	93.2
	78.47	94.5	80.00	96.4
	80.39	96.9	81.96	98.7
	82.02	98.8	83.62	100.7
	81.53	98.2	83.12	100.1
	80.16	96.6	81.73	98.5
	77.79	93.7	79.31	95.6
	81.49	98.2	83.08	100.1
	81.22	97.9	82.80	99.8
	81.58	98.3	83.17	100.2
Ave.	80.06	96.5	81.62	98.3

because it gave as high recoveries of oil as did the MgO-Super Cel mixture and did not in any case allow the emulsifier to pass through the column.

Two emulsions containing known amounts of oil B were prepared using commercial emulsifiers. One, Sample 1, was of the "miscible oil" type and contained 97.00 per cent of oil; the other, Sample 2, was of the "mayonnaise" type, and contained 83.00 per cent of oil and about 16 per cent of water. These were treated by the method, using silicic acid as the adsorbent and benzene-petroleum ether (1+4) as the solvent, drying the recovered oil one hour on the water bath. Results are given in Table 4.

The average percentage recovery of oil was 97.7 for Sample 1 and 96.5 for Sample 2. Corrected for the known fact that only 98.09 per cent of oil B would pass through a silicic acid column, the average percentage recoveries from the emulsions were 99.6 for Sample 1 and 98.3 for Sample 2.

DISCUSSION OF RESULTS

The question as to the accuracy and precision of the chromatographic adsorption method for the determination of mineral oil in its emulsions involves the question of just what "mineral oil" is. Petroleum oils are not of course individual compounds, nor are commercial spraying oils mixtures of pure hydrocarbons and nothing else. The chromatographic adsorption method has the advantage or disadvantage that it removes not only added emulsifiers but also some of the coloring matter and other minor ingredients of the oil. (It was shown in last year's report that material of a waxy nature was retained on passage of a petroleum ether solution of oil through a magnesia-Super Cel column but was eluted with alcohol.) The practical question that must be answered in devising a method for checking the guaranties for oil in commercial emulsions is how "oil" shall be defined for these purposes. It is our belief that to the minds of entomologists recommending oil sprays and farmers using such sprays "oil" means (so far as they consider the subject at all) the whole product as sold by the manufacturer minus any added emulsifier, and does not mean only the hydrocarbon content of the product.

It must also be taken into consideration that the number of emulsifying agents on the market is legion. It would probably be impossible to ascertain just what emulsifying agents were used in each of the emulsions now on the market, and if this information could be obtained it might be out of date by the time it was published. However, this fact does not necessarily make the task of devising a general method for determining mineral oil in its emulsions hopeless or even difficult, because all of these emulsifiers must have certain chemical properties in common for them to function as emulsifiers for petroleum oils. It would be expected that a chromatographic method would be at least as efficient a method for separating emulsifiers in general as would the older methods that use strong alkali, alcohol and centrifuging.

The present studies have shown that the chromatographic method, as applied to several different commercial oils and emulsions, is rapid and can yield results that are probably at least as reproducible as those obtained by the more usual methods employing a Babcock bottle (note that a Babcock bottle cannot be read to much closer than 0.1 per cent). These results are about 3 per cent low when "oil" is defined as commercial petroleum oil. Possibly the fact that oil is underestimated by about 3 per cent is unimportant for a determination of this type, and possibly a general correction factor could be used to correct the determined figures with sufficient accuracy. The method should be compared with one of the Babcock bottle methods[4] using several different emulsions of known oil content.

RECOMMENDATIONS*

It is recommended that the method as outlined, using silicic acid as the adsorbent and benzene-petroleum ether (1+4) as the eluant, be compared collaboratively with one of the Babcock bottle methods, on emulsions of known oil content.

REPORT ON BENZENE HEXACHLORIDE

By C. V. Bowen (Bureau of Entomology and Plant Quarantine, Agricultural Research Administration. U. S. Department of Agriculture, Beltsville, Md.), *Associate Referee*

An Industry-Government study to consider analytical methods for benzene hexachloride was initiated in March 1947 by the Chairman of the Interdepartmental Committee on Pest Control at the request of the Agricultural Insecticide and Fungidide Association. At the time there was no Associate Referee on benzene hexachloride. Considerable progress was made in this cooperative study toward the development of several satisfactory analytical methods and at the third conference, held in June 1949, it was agreed to cooperate with the Association of Official Agricultural Chemists in the study of methods to be presented in this report. Some of the studies of the Industry-Government group made possible the work presented herein.

The development of methods of analysis for benzene hexachloride is complicated by the fact that there are five known isomers as well as small amounts of other chlorinated products of benzene present in the technical product. Since the gamma isomer is much more toxic to insects than the other four, analysis for the total benzene hexachloride is of limited value. When residues or mixtures of benzene hexachloride with other materials are examined, determination of the total benzene hexachloride

[4] See, for example, California Dept. Agr. Bur. Chemistry Special Publication 229, "Economic Poisons, 1947–1948," pp. 7–9.

* For report of Subcommittee A and action of the Association see *This Journal*, 33, 39 (1950).

present may sometimes be adequate, but when the material is being assayed for insecticidal value it is essential to determine the percentage of gamma isomer present. The chemical behavior of the isomers is so nearly the same that attempts to develop analytical methods based on chemical reactions have not been fruitful. Only one method of analysis for the gamma isomer based on a chemical reaction has been proposed; most of the methods in use are based on physical measurements. Since this is the first report on benzene hexachloride, a brief survey of the methods proposed for total benzene hexachloride and for the gamma isomer is presented here.

DETERMINATION OF TOTAL BENZENE HEXACHLORIDE

(1) The determination of total organic chlorine is useful in the analysis of preparations of benzene hexachloride provided no other chlorine-containing compounds are present. In 1909 Bacon (1), in a study of the determination of total chlorine in organic compounds, determined the total chlorine in benzene hexachloride by refluxing the sample with ethyl alcohol and sodium. He found 73.23 per cent of chlorine, whereas the theoretical content is 73.18 per cent. A similar procedure using isopropyl alcohol has been very useful for the determination of DDT (2, 3). The success with DDT has led to the use of the same method for benzene hexachloride. The factor for benzene hexachloride from total chlorine is 1.3665.

(2) Total benzene hexachloride may also be calculated from the hydrolyzable chlorine obtained on dehydrochlorination by an alkali. Goldenson and Sass (4) determined benzene hexachloride in impregnated cloth by extracting the cloth sample with acetone in a Soxhlet apparatus, dehydrochlorinating the extracted benzene hexachloride with 0.3 *N* sodium hydroxide, and titrating the liberated chloride. The slightly low recoveries of 97 to 98 per cent were attributed to the presence of the beta isomer, which is resistant to alkaline dehydrochlorination (5).

Howard (6) in 1947 reported on the analysis for small amounts of benzene hexachloride by a turbidimetric determination of the liberated chloride following a dehydrochlorination by ethanolamine. This method was proposed for the analysis of insecticide deposits and of residues in foods. It was stated that with a 50-ml. Nessler tube "the eye can detect a turbidity due to 5 micrograms of chloride ion equivalent to 14 micrograms of benzene hexachloride." With a 100-g sample this would be equivalent to 0.14 p.p.m.

Barlow (7) used alcoholic potassium hydroxide to effect dehydrochlorination of benzene hexachloride that had been extracted from the blood of cattle. He used a modified Volhard procedure to determine the liberated chloride.

The factor for benzene hexachloride determined from chlorine removed

by dehydrochlorination with alkali is 2.7330. As can be seen by a comparison of the factors given for calculating the total benzene hexachloride from total chlorine and from hydrolyzable chlorine, the ratio is 1:2.

If water-soluble chlorine compounds are present in a technical product, they should be either removed before analysis or corrected for by means of a blank determination. Aside from the discrepancy caused by the beta isomer in the dehydrochlorination, the total benzene hexachloride determined from the total chlorine and by the dehydrochlorination method should give comparable results and may be used as checks against each other. Neither of these methods alone is of value unless it is known that benzene hexachloride is the only chlorine-containing material present.

Davidow and Woodard (8) have developed a method for determining small amounts of total benzene hexachloride based on the fact that the dehydrochlorinated product possesses a characteristic absorption band in the ultraviolet range. This method should find use in residue and tissue analysis, but does not at present appear to be adaptable for technical and formulated benzene hexachloride.

DETERMINATION OF THE GAMMA ISOMER

1. The first analyses for the gamma isomer of benzene hexachloride, reported by Slade (9), were made by fractional extraction and selective precipitation by taking advantage of the differences in solubility of the isomers in different solvents. The gamma and delta isomers were first separated from the alpha and beta isomers by dissolving them out from technical benzene hexachloride with a small amount of methanol, in which the latter two isomers are practically insoluble, and then recrystallizing the gamma isomer from chloroform. The isomers were identified by their melting points and their purity was confirmed by lack of change in melting point on further recrystallization. The separated isomers were then weighed. This method is laborious and time consuming as well as lacking precision.

2. Use of the infrared spectrometer (10) was the first practical method employed in determining the percentages of the isomers present. The apparatus is expensive, however, and the qualitative composition must be known before a sample can be analyzed. Although this method has been used in England for several years, Daasch (11) published in 1947 the first detailed procedure for the infrared spectroscopic analysis of the five isomers known to be present in technical benzene hexachloride. He concluded that "the method is entirely satisfactory for the analysis of nearly pure samples of the insecticide and is relatively free of interference from the usual type of impurities encountered."

3. Ramsey and Patterson (12) successfully used partition chromatography as a means of separating technical benzene hexachloride into its components. A known mixture of the alpha, gamma, and delta isomers

was separated in like manner. Aepli, Munter, and Gall (13) have developed this procedure into an analytical method for the determination of the gamma isomer so that one operator can make four determinations in an 8-hour day. Nitromethane and *n*-hexane are used as the partition solvents, and silicic acid is the supporting solid of the column. The accuracy is said to be about 2 per cent based upon the gamma isomer content. Recently Harris (14) has modified this method by adding a dye to locate visually the position of the gamma isomer as it moves down the column and thus reduce the number of fractions that it is necessary to collect.

4. A cryoscopic method of analysis (15) for the determination of the gamma isomer has been developed. It is based upon the fact that the freezing point of a pure compound (in this case the gamma isomer) is lowered by the presence of dissolved materials. When inert diluents are present, they must be removed before the benzene hexachloride can be analyzed for its gamma isomer content by this method. This method is useful for the determination of the gamma isomer in products of high gamma isomer content but is of less value for the analysis of the usual technical grade. The cryoscopic method may prove to be useful for determining the purity of lindane.

5. The only chemical method for determining the gamma isomer content of benzene hexachloride materials is that of LaClair (16). The method is based on the differences in the rates of dehydrochlorination of the several isomers at 0°C. by alcoholic potassium hydroxide. Under the reaction conditions the beta isomer is inert and the alpha and delta isomers are almost completely dehydrochlorinated before the gamma isomer is appreciably acted on. If the epsilon isomer content is low enough not to interfere seriously, the difference in dehydrochlorination in a short time and in a longer time, *e.g.* 15 and 50 minutes, can be related to the gamma isomer content. The method does not apply to materials containing over 70 percent of the gamma isomer. The temperature and length of the dehydrochlorination periods are critical.

6. In 1946 Keller *et al.* (17) reported that benzene hexachloride can be reduced at a dropping mercury electrode at a potential of -1.83 volts. Ingram and Southern (18) observed that the gamma isomer in an aqueous ethanol solution gave a wave for a progressive reduction over the range of -1.15 to -1.55 volts against a saturated calomel electrode. After the preparation of wave height-concentration curves, analyses could be made by the comparison method. The other isomers did not give reduction waves. Dragt (19) has given a more detailed procedure for the polarographic determination of the gamma isomer in an aqueous acetone solution with a potassium chloride-sodium acetate buffer solution. The method is said to possess a precision of ± 0.5 per cent of the gamma somer.

7. A mass-isotope dilution method (20) for the determination of the gamma isomer of benzene hexachloride has been developed in which

gamma hexadeuterobenzene hexachloride is used as a tracer molecule and the extent of the dilution determined by use of infrared spectroscopy. This method is theoretically a basic method which is unaffected by the presence of impurities.

COLLABORATORS

The following collaborators took part in this study:

Aepli, O. T. Pennsylvania Salt Manufacturing Co., Philadelphia, Pa.
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A critical study of the methods reviewed above with regard to (a) time required, (b) availability of apparatus and reagents, and (c) apparent accuracy led to a decision to investigate the partition chromatographic method and compare this with other methods available to the collaborators. The fact that the infrared spectrometric method is used by a number of laboratories suggested that these two methods should be compared.

Each collaborator was furnished with a reprint of the original partition chromatographic method (13), a copy of the procedure using a dye marker (14), and a copy of the tentative A.O.A.C. method for the determination of total chlorine in DDT. He was requested to give his opinion as to the use of the total-chlorine procedure for the determination of total benzene hexachloride. One collaborator reported the finding of total benzene hexachloride calculated from total chlorine as follows:

Sample	Per cent
1	99.7
2	101.3
3	100.1
4	99.0
5	100.2

Sample 1 was purified benzene hexachloride; the others were of the technical material.

These results are in agreement with those obtained by Bacon (1), who in 1909 obtained 72.23 percent of total chlorine, which calculated to benzene hexachloride gives 100.07 per cent. It is well to remember that the presence of higher chlorinated products, such as heptachlorocyclohexane and octachlorocyclohexane, will give high results, whereas the presence of the chlorine-substituted benzene derivatives aside from hexachlorobenzene will give low results.

The following were also sent to each collaborator:

50 mg. of D and C Violet Dye No. 2. (1 hydroxy-4-*p*-toluinoanthraquinone)
2 g of alpha isomer of benzene hexachloride
2 g of gamma isomer of benzene hexachloride

Three samples designated as:
No. 1. Technical benzene hexachloride
No. 2. Dust formulation containing less than 10 per cent of the gamma isomer

TABLE 1.—*Analysis of synthetic mixtures by partition chromatography*

CHEMIST[a]	GAMMA ON COLUMN MG	PER CENT GAMMA			
		SYNTHETIC NO. 1		SYNTHETIC NO. 2	
		KNOWN	FOUND	KNOWN	FOUND
1	100	10.3[b]	10.3	—	—
2	148.8	10.0	9.95	—	—
	154.3	—	—	51.0	51.3
3	—	10.0	10.37	—	—
	—	—	—	50.0	47.93
4	—	10.0	10.48	—	—
	—	—	—	50.0	50.7
5	—	10.2	9.99	—	—
	—	—	—	50.3	49.2
				49.5	49.6[c]
6	—	—	—	50.0	49.29
7	16.0	10.0	10.0	—	—
	34.7	—	—	50.0	50.5
8	—	—	—	61.2	60.6
9	—	10.0	10.1	—	—
	—	—	—	50.0	50.3

[a] Does not conform with list of collaborators as previously given.
[b] Contained also 80.1% of alpha, 2.4% of beta, and 7.2% of delta isomers.
[c] Aepli extraction procedure.

TABLE 2.—*Analysis of synthetic mixtures by infrared spectroscopy*

CHEMIST[a]	PER CENT GAMMA			
	SYNTHETIC NO. 1		SYNTHETIC NO. 2	
	KNOWN	FOUND	KNOWN	FOUND
1	10.0	10.2	50.0	49.7
2	10.0	10.3	50.0	50.3

[a] Designation does not conform with the order of names or numbers as previously given.

No. 3. Wettable-powder formulation containing more than 25 per cent of the gamma isomer.

The analyses were to be made by the dye marker modification (14) of the original partition chromatographic method (13). The collaborators were instructed to prepare and run two synthetic mixtures of the alpha and gamma isomers, of 50 and 10 per cent gamma content, before analyz-

TABLE 3.—*Analysis of A.O.A.C. samples for the gamma isomer of benzene hexachloride by partition chromatography*

CHEMIST[a]	SAMPLE NO. 1		SAMPLE NO. 2		SAMPLE NO. 3	
	GAMMA ON COLUMN	GAMMA	GAMMA ON COLUMN	GAMMA	GAMMA ON COLUMN	GAMMA
	mg	*per cent*	*mg*	*per cent*	*mg*	*per cent*
1	—	14.97	—	1.68	—	50.7
	—	14.54[b]	—	—	—	—
2	—	11.2	—	1.2	—	48.6
					—	50.0[b]
3	139.8	14.0	74.0	1.48	149.4	49.6
4	40	12.5	60.0	1.3	240.0	47.8
	125	12.3	—			
5	—	12.5	—	—	—	40.0
6	36.2	12.9	—	—	—	—
7	25.2	16.8	7.6	1.86	—	53.0
8	—	13.32	—	1.54	—	43.75
9	—	13.05	—	1.51	—	50.35
10	—	12.85	—	1.56	—	50.05

[a] Designation does not conform with the order of names or numbers as previously given.
[b] Aepli method of extraction

ing the samples. One collaborator had the mixture of pure isomers approximating 50 per cent prepared by a disinterested person, while two others used samples of approximately 50 and 10 per cent gamma isomer content. The results as given in Table 1, together with the data obtained by Aepli *et al.* (13), show the reliability of the method. Comparison of Tables 1 and 2 also supports the reliability of the method.

As a chemist becomes familiar with the partition chromatographic method he will obtain more nearly correct results. The results in Table 3 tend to be high when the amount of gamma isomer on the column is small (chemist 7). G. S. Haines says that "the sample size should be adjusted to provide 100 to 125 mg. of benzene hexachloride on the column to minimize errors in weighing and transfer." Low results in the analysis

TABLE 4.—*Analyses of A.O.A.C. samples by other methods*

CHEMIST[a]	SAMPLE NO. 1	SAMPLE NO. 2	SAMPLE NO. 3
	Infrared Spectroscopy		
1	13.0	1.5	51.5
2	13.5	1.14	50.4
3	13.2	1.4	50.2
4	13.2	1.9	—
	Polarography		
5	13.8	1.8	44.8
6[b]	13.4	1.54	50.7

[a] Designation does not conform with the order of names or numbers as previously given.
[b] Polarograph standardized against infrared spectroscope.

of sample No. 3 were obtained in some cases. This discrepancy is probably due to insufficient extraction of the sample, since there were five good checks for 50 per cent by this method and comparable results were obtained by infrared spectroscopy.

The infrared spectroscopic method shows consistent results (Tables 2 and 4) for both the synthetic mixtures and the unknown samples, although one result, 51.5, appears to be somewhat out of line. The wetting agent used in this wettable powder may influence the results obtained by this method.

Two of the collaborators analyzed the samples by the polarographic method (Dragt, 19). The low result is probably due to incomplete extractions.

In cases where the short column used by Harris (14) does not give a clean separation of the gamma isomer, the longer column of Aepli *et al.* (13) should be used.

P. A. Clifford states that it is advisable, when weighing up the collected gamma isomer, to use an Erlenmeyer flask of slightly less weight as a tare.

In order to eliminate errors care should be taken (a) to have complete

extraction of the sample, (b) to use calibrated apparatus in obtaining exact aliquot for chromatographing, and (c) to maintain the sample solution at near the same temperature for the measuring of all aliquots.

The Associate Referee acknowledges the cooperation and the assistance of the collaborators, and the assistance of J. F. Gall, J. F. Garrett, and Horace E. Hall.

RECOMMENDATIONS*

It is recommended—

(1) That the modified partition chromatographic method (14) using the procedure for the preparation of the sample as given in the original method (13) and the infrared spectrometric method (11, 21) be adopted by official first action as alternative methods for the determination of the gamma isomer in technical benzene hexachloride.

(2) That the modified partition chromatographic method as given above be adopted by official first action for the determination of the gamma isomer of benzene hexachloride in wettable powder and dust insecticidal formulations.

(3) That the investigation of the analysis for the gamma isomer of benzene hexachloride in emulsion concentrates, solutions, and formulations containing other organic insecticides be continued.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **33**, 39 (1950).

REPORT ON PARATHION

By FRED I. EDWARDS, (U. S. Department of Agriculture, Agricultural Research Administration, Bureau of Entomology and Plant Quarantine, Beltsville, Md.), *Associate Referee*

Work done during World War II by Gerhard Schrader, a German chemist employed by I. G. Farbenindustrie (1, 2), first demonstrated the insecticidal possibilities of organo-phosphorus compounds. Parathion, originally designated by Schrader as E-605, is at present one of the most promising of these organo-phosphorus insecticides. It has been found highly effective against a long list of insect species (3, 4), even when applied in extremely low concentrations.

Parathion is the accepted trivial name for the compound, O, O-diethyl O-*p*-nitrophenyl thiophosphate. The pure compound is a pale yellow, practically odorless oil, which crystallizes into long needles melting at 6.0°C. (5). Samples purporting to be technical parathion have been found on analysis to contain from as little as 1 per cent to practically 100 per cent, depending on the efficiency of manufacture. Generally, technical parathion analyzes about 90–94 per cent.

Parathion is soluble in most organic solvents and is only slightly soluble in water (ca 20 p.p.m. at 20°C.). Like many other organic phosphorus compounds, parathion has a high boiling point and, since it undergoes thermal decomposition, can be distilled only under high vacuum (6). The hydrolysis rate of parathion has been measured by Peck (7), who found that at a *p*H of 10 or less the time for 50% hydrolysis at 25°C. is 120 days; in a solution of saturated lime water the time is 8 hours.

Parathion is an inhibitor of cholinesterase, an enzyme generally present in nervous tissue. Its biological activity can probably be largely accounted for on the basis of this enzymatic inhibition (8). Current work on toxicity indicates that extreme care must be exercised in the handling of parathion (9) and this should be kept in mind in analyzing this material.

Methods of analysis for technical parathion and its dust formulations have not previously been studied by this Association and this report will therefore review the methods currently in use and under development.

METHODS

METHOD 1—COLORIMETRIC DETERMINATION

A method developed by Averell and Norris (10) for the determination of spray residues of parathion on plant materials has been applied by several collaborators for assaying this material. The method is carried out in three steps (1) reduction with zinc and hydrochloric acid to form an amino compound (2) diazotization by adding sodium nitrite to the acid solution, and (3) coupling with N-(1-naphthyl) ethylenediamine to produce a magenta color which may be measured in a colorimeter. The

procedure is suitable for amounts of parathion from 20 to 200 micrograms. The method is rapid and simple. It is, however, subject to interference from a number of compounds. The Associate Referee has tested two major contaminants of commercial parathion (11), O-ethyl O, O-bis (*p*-nitrophenyl) thiophosphate and *p*-nitrophenol, by this method. *p*-Nitrophenol does not interfere under the conditions specified by Averell and Norris (10), but O-ethyl O, O-bis(*p*-nitrophenyl) thiophosphate gives a color that has an absorption curve identical with that given by parathion. Six other aromatic nitro compounds (*m*-nitrophenol, *p*-nitrotoluene, *o*-nitrotoluene, diethyl *p*-nitrophenyl phosphate, 4-nitrobiphenyl, and *o*-nitrophenol) were also subjected to the conditions of the method. Of these six compounds the first four gave a magenta color identical with that of parathion; the fifth gave a bluish purple color, which would definitely interfere. The sixth compound did not develop color in the 10-minute period specified in the method, but in one hour had produced a purple color similar to the magenta one of parathion.

Method 2—Polarographic Determination

Bowen and Edwards (12) developed a method for the determination of parathion by means of the polarograph. 1:1 solution of acetone and water containing potassium chloride as an electrolyte, acetic acid as a buffer, and gelatin as a suppressor is used. The method requires a final aliquot containing about 10 milligrams of parathion. As developed the method calls for an expensive apparatus, but it can be adapted to use a non-recording, manually operated polarograph, which is less expensive. *p*-Nitrophenol does not interfere with the parathion polarogram. O-ethyl O, O-bis (*p*-nitrophenyl) thiophosphate gives a wave at a voltage very close to that of parathion. If present as an impurity it will therefore interfere, adding to the parathion wave height.

Method 3—Ultraviolet Absorption

An unpublished method developed by Gisclard and Hirt (13) for determining parathion vapors in air was investigated for possible use in assay work. Utilization is made of the fact that parathion has an ultraviolet absorption peak at 274 millimicrons. The solvent used is 95% ethyl alcohol. The method requires a spectrophotometer with ultraviolet adapters and quartz cells. Here again the cost of such apparatus is high. The method is extremely sensitive, and will detect parathion at a concentration of from 1 to 10 micrograms per milliliter of solution. The dilutions necessary to achieve this final concentration would introduce a large factor of error. Interference from *p*-nitrophenol can probably be in great part eliminated by taking a second reading at 310 millimicrons, which is the absorption peak of *p*-nitrophenol. The interference from O-ethyl O, O-bis (*p*-nitrophenyl) thiophosphate poses a more difficult problem. Its absorption curve so closely follows that of parathion that it does not seem possible to distinguish between the two compounds.

Method 4—Combination Titration and Color Development

A method utilizing a two-step determination consisting of a colorimetric determination and a titration has been developed (14) but has not yet been published. The colorimetric step consists of washing an ether solution of parathion with aqueous sodium carbonate to remove free *p*-nitrophenol. This wash solution of sodium nitrophenolate is diluted to a standard volume and its concentration evaluated with a colorimeter. The second step consists of the reduction of the ether soluble portion of the sample with zinc and hydrochloric acid and a titration of the resulting amino compound with standard sodium nitrite solution at 0°–5°C. The end point is determined by using potassium iodide-starch paper to detect the point at which free nitrite ions are present. This titration will give a percentage figure for the amino groups present, which may be expressed as per cent parathion present. The presence of any other aromatic nitro compound not affected by the alkali wash will cause error. Any O-ethyl O, O-bis (*p*-nitrophenyl) thiophosphate present will give an apparent parathion value that will be doubled, because the compound contains two nitro groups which will be reduced and diazotized. The method has in its favor the low cost of equipment and its simplicity.

Method 5—Distillation

A method utilizing a distillation at very low pressures has been described by Thomson (15). As in method 4, the free *p*-nitrophenol is determined colorimetrically and subsequently subtracted from the distillation result. Since parathion is unstable under heat, the method may be subject to error through decomposition. More important is the fact that the method is potentially dangerous and extreme caution must be used during the heating. Various reports have been received describing explosions resulting from heating parathion. Thomson repeatedly cautions against overheating and warns that explosions may result.

METHODS FOR DUSTS

In the discussion of methods thus far no mention has been made of the analysis of dusts. There is no essential difference from the foregoing procedures after parathion has been extracted from the dust. Three methods of extraction have been reported by collaborators. Two collaborators are using a flask extraction wherein a sample of dust is placed in a volumetric flask with ethyl alcohol, shaken intermittently for one hour, and an aliquot taken for analysis. One collaborator is using a similar flask extraction with acetone. A fourth collaborator is extracting with ether in a Soxhlet apparatus. In connection with the development of a polarographic method (12), the Associate Referee carried out extractions by flask and Soxhlet methods and subsequent polarographic analysis showed no difference in recovery of parathion. It appears that any one of these methods is satisfactory.

COMMENTS OF COLLABORATORS

In general the comments of the collaborators indicate dissatisfaction with the methods they are using. Main objections are centered around the interference of impurities containing the nitro group, which lead to high results. All methods reviewed are subject to criticism on this point.

COLLABORATORS

The assistance of the following collaborators is gratefully acknowledged:

Aepli, O. T., Pennsylvania Salt Manufacturing Co., Wyndmoor, Pa.
Averell, P. R., American Cyanamid Co., Stanford, Conn.
Lemmon, A. B., California Department of Agriculture, Sacramento, Calif.
Sunderlin, R. S., Eston Chemicals Inc., Los Angeles, Calif.
Thomson, H. A., Naugatuck Chemicals, Elmira, Ont.
Towne, R. W., Monsanto Chemical Co., St. Louis, Mo.
Zabor, J. W., Pittsburgh Coke & Chemical Co., Pittsburgh, Pa.

RECOMMENDATIONS*

It is recommended that all methods which show promise be investigated with the view of removing interference from other nitro compounds that may be present in technical parathion.

ADDENDUM

Since the preparation of this report a modification of one method discussed and one new method have been made pubic. No experimental work has been carried out on these methods and full details on them are not known. Such information as is available is given in the following descriptions.

Method A.—Gage (16) has modified the procedure of Averell and Norris (10) with respect to solvent and reagents and reports that improved determinations have resulted. He uses toluene as the solvent and does not remove it before carrying out the analysis. The parathion, in solution with toluene, is reduced to its amino derivative with zinc and acetic acid. The amino compound is extracted into dilute hydrochloric acid, diazotized, and coupled with *N*-sulphatoethyl-*m*-toluidine. The resultant color is evaluated by means of a colorimeter. The question of interference by impurities occurring in technical parathion is covered by the author only in regard to O-ethyl, O, O-bis(*p*-nitrophenyl) thiophosphate which he states will interfere. He circumvents this by an indophenol reaction using o-cresol and sodium hydroxide to produce a blue color for colorimetric evaluation.

While the O-ethyl O, O-bis (*p*-nitrophenyl) thiophosphate does not interfere in this reaction, free *p*-nitrophenol does. To circumvent this interference another indophenol reaction is run using o-cresol and ammonia.

* For report of Subcommittee A and action of the Association, see *This Journal*, 33, 40 (1950).

This gives a figure for free *p*-nitrophenol which may be subtracted from the previous overall figure.

Method B.—Ketelaar (17) has developed a method based on the determination of total and free *p*-nitrophenol. Free *p*-nitrophenol is determined by extraction with a 1% soda solution and colorimetric evaluation of the resulting yellow solution. Total *p*-nitrophenol is determined by hydrolysis with boiling alcoholic sodium hydroxide and colorimetric evaluation of the resulting solution. The amount of bound *p*-nitrophenol is taken as the difference between these figures. No mention is made of errors introduced by impurities occurring in technical parathion.

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REPORT ON ORGANIC THIOCYANATES

By H. A. ROONEY (Bureau of Chemistry,* State Department of Agriculture, Sacramento, Calif.), *Associate Referee*

Organic thiocyanates are commonly used in agricultural and household insecticides. Determination of these compounds from total nitrogen or total sulphur is satisfactory only when no other nitrogen or sulphur compounds are present. J. W. Elmore developed a method (*This Journal*, **28**, 363 (1945)) in which a potassium polysulphide reagent converts organic thiocyanates to inorganic thiocyanates. Cuprous thiocyanate is then

* Allen B. Lemmon, Chief.

precipitated and a Kjeldahl determination of nitrogen permits calculation of the organic thiocyanate nitrogen content of the original material.

The writer was appointed Associate Referee to conduct a collaborative investigation of this method, but he was unable to secure collaborators for this project.

There is no official method for analysis of these important insecticides and there is real need to determine if the Elmore method or a modification of it is sufficiently accurate and precise when used by different laboratories. It is recommended that an effort again be made to secure collaborators for the work.

REPORT ON DETERMINATION OF DITHIOCARBAMATES

By J. D. PATTERSON, (State of Oregon, Dept. of Agriculture Salem, Oregon) *Associate Referee*

Various derivatives of dithiocarbamic acid have been recommended as plant fungicides. Among those found commercially are the iron salts principally as ferric dimethyldithiocarbamate, the zinc salts as zinc ethylene bis-dithiocarbamate and zinc dimethyldithiocarbamate and finally the sodium salt as disodium ethylene-bis-dithiocarbamate. Their growing use in the formulation of insecticides and fungicides has brought about the need for an accurate and if possible simple method of analysis.

The Associate Referee has considered the following possible approaches to the problem:

1. The determination of iron, zinc, sulfur, or nitrogen with the results calculated to the equivalents of the dithiocarbamate sought (5).
2. The direct colorimetric estimation of the iron salt (3).
3. The determination of carbon disulphide liberated from the dithiocarbamates when treated with dilute acid either by a colorimetric method (1, 2) or by an iodimetric method (4).

DISCUSSION

The analysis of the dithiocarbamates by method No. 1, *i.e.* via iron, zinc, nitrogen, or sulfur, in most cases at least, does not prove desirable because of contaminating residues and additives. Further, because of the instability of these compounds as evidenced particularly by the presence of free sulfur, a report on the basis of these elements would probably prove erroneous. The following results were obtained by the use of the above method (6):

	Ferric dimethyl	*Dithiocarbamate*
Guarantee	*Calculated from iron*	*Calculated from nitrogen*
70%	95.90%	66.20%
70%	102.25%	64.72%
	Zinc dimethyl dithiocarbamate	
Guarantee	*Calculated from zinc*	*Calculated from nitrogen*
70%	81.60%	80.40%
70%	75.90%	78.16%

Method No. 2 or the direct colorimetric estimation is applicable only to the iron salt and then only in microquantities. No serious attempt was made to investigate it further.

Method No. 3 it seems may be the most desirable approach by reason of the fact that CS_2 is characteristic to the molecule of all the various dithiocarbamate salts. Further it is a volatile liquid that should cause little trouble as a possible impurity due to decomposition. The colorimetric estimation of CS_2 was not investigated since here again the authors of the method held it to be only of value for the analysis of micro amounts. However, the iodometric method of Cullan and Strafford (4) reported as applicable to certain dithiocarbamates used in the rubber industries seemed to offer a more practical approach. It is hoped that a way may be found to apply their method to the materials used as fungicides. Briefly their procedure is as follows:

The sample is digested with dilute acid in all glass apparatus. The CS_2 evolved is passed over into alcoholic potassium hydroxide where potassium xanthate is formed according to the following reaction:

$$CS_2 + C_2H_5OH + KOH = KS.CS.OC_2H_5 + H_2O$$

The xanthate alcoholic potassium hydroxide solution is then neutralized and made just alkaline with a slight excess of sodium bicarbonate and then titrated with standard iodine. The probable reaction is as follows:

$$2KS.CS.OC_2H_5 + 2I = S_2(CSOC_2H_5)_2 + 2HI$$

It became apparent at once that the procedure as outlined resulted in a fleeting starch-iodine end point. Work done in our laboratory has shown that this condition may be controlled somewhat by dilution of the solution before titration. Then it was noted that even a slight excess of sodium bicarbonate caused more rapid fading. By neutralizing carefully to a phenolphthalein end point and immediate titration this interfering material was eliminated. The xanthate breaks down slowly in neutral or slightly acid media, however, and may give low results unless the titration is carried out rapidly. If the solution to be titrated is cooled to 0°C. a sharp end point is obtained even in the presence of sodium bicarbonate.

It has been noted throughout the investigation that other materials were distilled simultaneously with the CS_2. In studying the nature of this decomposition certain other interesting facts were brought out. First, when the dithiocarbamates were boiled with distilled water, and the distillate collected in Alc-KOH a slow but definite evolution of CS_2 was noted. It might be suspected then that the dithiocarbamates break down in the presence of atmospheric moisture, CS_2 being one of the products of decomposition. Likewise a yellow amorphous material passing over with the distillate was shown to be elemental sulfur, again indicating a product of decomposition. Along with the materials sulfur

and CS_2 a compound having a disagreeable odor is most evident. The nature of this material is not known but in attempts to separate it from the CS_2 by trapping, it was found in the water distillate and therefore could be held by a simple receiver water trap. There seemed to be no appreciable loss of CS_2 by the inclusion of the trap in the system, and likewise it was discovered that by eliminating this water soluble material, the problem of the fading end point was completely solved. The blue starch-iodine color was stable for several hours.

At present the CS_2 procedure still lacks the refinement necessary for an acceptable method.

Sample results obtained on Ferric dimethyl dithiocarbamate, extracted from commercial samples and twice recrystallized from chloroform gave recoveries as follows:

From Iron	*From Nitrogen*	*From Sulfur*	*Carbon Disulfide*
98.59%	97.79	97.30	94.35
98.76%	96.31		93.50

Future investigation plan is to consider the nature of the breakdown products occurring under conditions of the carbon disulfide determination. Work to date has been done in presence of air; it is proposed to use nitrogen to avoid possible atmospheric oxidation. Also methods of purification of pure reference compounds require more investigation.

The Associate Referee recommends* that the investigation be continued.

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(6) Communication from Allen Lemmon, Bureau of Chemistry, Calif. State Dept. Agric.

The contributed paper entitled "Determination of Gamma-Benzene Hexachloride in Insecticides," was published in the August number (1949) of *This Journal*, page 684.

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* For report of Subcommittee A and action of the Association, see *This Journal*, **33**, 39 (1950)

REPORT ON DISINFECTANTS

By L. S. STUART (Insecticide Division, Livestock Branch, Production and Marketing Administration, U. S. Department of Agriculture, Washington, D. C.), *Referee*

Changes are proposed as given below in the method *Phenol Coefficient (44)-Official* as it appears under Chapter 6, *Insecticides and Fungicides*, in the Sixth Edition (1945).

The name *Eberthella typhosa* in the sub-heading I., and **6.153(b)** and the abbreviation *Eb. typhosa* in **6.156** should be changed to *Salmonella typhosa* and *S. typhosa*, respectively. Also, the name *Staphylococcus aureus* and abbreviation *Staph. aureus* in **6.157**, should be changed to *Micrococcus pyogenes* var *aureus* and *M. pyogenes* var *aureus*, respectively. These changes are necessary to bring the method into conformance with changes in the official classification of these species of bacteria made in the sixth edition, 1948, of Bergey's Manual of Determinative Bacteriology.

Under **6.153(b)** *Test organism.*—the first sentence should be revised to read: "The Hopkins strain 26 of *Salmonella typhosa* (Zopf) Weldin, F.D.A., A.T.C.C. No. 6539 (formerly called *Bac. typhosus* and *Eberthella typhosa*)." Also, under **6.157**, the third sentence should be changed to read: "Culture of *M. pyogenes* var *aureus* F.D.A., 209 A.T.C.C. No. 6538 (formerly known as *Staphylococcus aureus*) shall be used and must have at least the resistance indicated by the following:" These changes will be necessary as a result of the changes in nomenclature proposed and to provide a slightly more specific designation of the actual cultures to be employed.

Under **6.154**(d) *Transfer loop,*—the specification "Pt. wire" should be changed to read: "Pt or Pt alloy wire," since most laboratories are now using Pt. alloys of the Platinum-Ruthenium type for this work. The resulting loop is more rigid and easier to manipulate.

Under **6.155** Procedure—the following sentence should be inserted in the first line beneath Fig. 11 after the sentence ending " . . . 15 min. period." "Medicant tubes should be agitated gently before taking each interval loop sub-sample for transfer to subculture media." In this same section another sentence should be inserted to follow the sentence reading, "Incubate subcultures at 37°, 48 hours, and read results" to read as follows: "Individual subculture tubes should be thoroly agitated before incubation." Heretofore it has been assumed that adequate mixing of the medicant and subculture tubes could be taken for granted, but observations made by your Referee and various collaborators indicate that individual techniques vary considerably on this point. The revision suggested will make agitation at the points indicated mandatory, and thus promote greater uniformity of result.

Under **6.153(a)**—(1) the phrase, "and sterilize at 15 lbs. pressure 40 min." should be changed to read: "and sterilize at 15 lbs. steam pressure 20 min." The designation of a 40 min. sterilizing time in the Sixth Edition was apparently a typographical error.

The last sentence in **6.153(a)**—(1) should be changed to delete the phrase "and subcultures" and replace it with the phrase "of test cultures." A section (3) should be added under **6.153 (a)** Culture Media, to read as follows: "(3) *Subculture Media:* Use (A), (B), or (C) whichever gives the lowest result: (A) *Nutrient broth* described in (1): (B) Fluid Thio-glycollate Medium U.S.P., XIII: Mix 0.75 g of l-cystine, 0.75 g of agar, 2.5 g of NaCl, 5.5 g of dextrose, 5.0 g of water-soluble yeast extract,

and 15.0 g of pancreatic digest of casein with 1,000 ml of H_2O; heat to dissolve on water bath; add 0.5 g of sodium thioglycollate or 0.3 g of thioglycollic acid, and adjust with 1 *N* NaOH to *p*H 7.0±0.1; reheat without boiling and filter thru moistened filter paper; add 1.0 ml of 0.1 % Resazurin sodium soln freshly prepd.; tube in 10 ml quantities in 20×150 mm bacteriological test tubes; plug with cotton, and sterilize at 15 lbs steam pressure for 20 min.; cool at once to 25° and store at 20°–30°C.: (C) "Letheen broth:" Dissolve 0.7 g of lecithin (Azolectin) and 5.0 g of polyoxyalkylene derivative sorbitan monoöleate (Tween 80) in 400 ml of hot water; boil until clear; add 600 ml of soln of 5.0 g beef extract (Difco), 10.0 g of peptone (Armour), and 5 g of NaCl in H_2O; boil 10 min. and adjust with 1 *N* NaOH and/or 1 *N* HCl to *p*H 7.0±0.2; filter thru coarse filter paper; tube in 10 ml quantities in 20×150 mm bacteriological test tubes; plug with cotton, and sterilize at 15 lbs steam pressure 20 min. With oxidizing products and products formulated with toxic compounds containing certain heavy metals like Hg (B) will usually give the lowest result. With products containing cationic surface active material (C) will usually give the lowest result." These particular changes are designed to provide 3 alternatives in the selection of subculture media.

It can be said that if a test organism fails to grow when transferred to a suitable medium then and only then has it been killed. The provision of the 3 alternative subculture media described above will give greater assurance for use of a suitable medium in subculturing and this has become especially important in the testing of formulations wherein small amounts of surface active materials and organic salts of heavy metals possessing very high bacteriostatic effects but relatively low bactericidal activities are encountered either as bona fide ingredients or adulterants. Media (B) and (C) were selected as alternative for (A) "Nutrient broth" in subculturing after consideration of such factors as availability, ease of standardization, use history, effectiveness in overcoming bacteriostatic effects which are most commonly encountered, and results of collaborative studies.

Fluid Thio-Glycollate Medium U.S.P. XIII was developed especially for testing the sterility of biological preparations containing such preservatives as phenol, cresol U.S.P., merthiolate, phenyl mercuric acetate, phenyl mercuric borate, phenyl mercuric nitrate, chlorbutanol and formaldehyde. The use of sodium thioglycollate in a clear medium was proposed almost simultaneously by Brewer (1940) (*J. Bact.*, **39**, 10) and Linden (1941) (unpublished). The two formulas recommended differed only in minor detail. In 1941 the National Institute of Health Bulletin "Fluid Thioglycollate Medium for Sterility Test" appeared, designating a medium of this type as a replacement for infusion broth in sterility testing work. The formula recommended at that time was revised in an amendment to this bulletin issued on July 13, 1942, and following additional experimental work by Pittman (*J. Bact.*, **51**, 19, 1946) was again revised for use in the thirteenth edition of the U. S. Pharmacopoeia. It may be made from the individual ingredients as described, but is also available in dehydrated form prepared according to specification by biological supply houses. In addition to use for sterility testing it is widely

used as a general culture medium for propagating fastidious organisms. The recent work of Morton, North, and Engley (J. Am. Med. Assoc., 236, 36–41, 1948) established that the use of this medium in the primary subculture of bacteria exposed to various mercurial compounds gave a more reliable differentiation between bacteriostatic and bactericidal effects than the Shippen technique (*Am. J. Pub. Health*, 18, 1232–1234, 1928) employing primary and secondary subcultures in Nutrient broth. In these studies good correlation was secured between "in vitro" germicidal experiments with hemolytic streptococci when Fluid Thioglycollate Medium was used for subculturing and "in vivo" experiments with the same organism wherein fatal septicemia in mice was used as the index to germicidal activity.

As a part of a collaborative study conducted early in 1949 by the Disinfectant Scientific Committee of the National Association of Insecticide and Disinfectant Manufacturers tests were made to determine if results with a commercial brand of phenolic disinfectant containing soap, orthohydroxy diphenyl and cresylic acids as active ingredients by the A.O.A.C. Official Method, in which Fluid Thioglycollate Medium was substituted for nutrient broth in subculturing, could be reproduced as readily as in the official method.

The chairman of the committee directing these tests was A. R. Cade. The following individuals representing six testing laboratories acted as collaborators:

A. R. Cade (Givaudan-Delawanna, Inc.), Delawanna, New Jersey.
A. F. Guiteras and Miss R. L. Shapiro (Foster D. Snell, Inc.), New York City.
E. G. Klarmann and Mrs. E. S. Wright (Lehn and Fink Co.), Bloomfield, New Jersey.
H. E. Lind (Sias Laboratories), Brookline, Massachusetts.
R. S. Schumard, (Monsanto Chem. Company), St. Louis, Missouri.
L. S. Stuart and John Bogusky (Insecticide Division, Livestock Branch, Production and Marketing Administration, USDA, Washington, D. C.

Replicate samples of a widely distributed commercial phenolic type germicide were forwarded to the collaborators by Dr. Cade with instructions to test it 3 times in quadruplicate at 20°C. against *S. typhosa* and *M. pyogenes* var *aureus* at stipulated dilutions both by the official A.O.A.C. method and the official A.O.A.C. method modified in that Fuid Thioglycollate Medium was to be substituted for Nutrient broth in subculturing. Collaborators were requested to report the results in terms of the number of subculture tubes for the various dilutions tested showing growth and the number showing absence of growth at the 10 min. exposure interval. Thus, if a laboratory completed the study as initially outlined, the report would show a total of 12 determinations at the one exposure interval for each dilution by both procedures. Four out of the 6 collaborating laboratories completed this study as outlined and the other 2 submitted some data. The results are presented in Table 1:

TABLE 1.—*Comparison of results on replicate determinations at the 10 min. exposure interval of phenolic disinfectant secured by official procedure and official procedure modified in that fluid thioglycollate medium was used in lieu of nutrient broth in subculturing*

TEST ORG.	DILUTION	COLLABORATING LABORATORIES BY NUMBERS																					
		1				2				3				4				5				6			
		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE	
		−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+	−	+
Eb. typhosa	1–400	12	0	9	3	5	7	4	8	4	8	4	8	6	0	6	0	10	2	10	2	2	0	1	1
	1–450	7	5	0	12	2	10	2	10	3	9	4	8	6	0	6	0	9	3	2	10	1	1	1	1
	1–500	0	12	0	12	0	12	0	12	0	12	0	12	6	0	6	0	9	3	0	12	1	1	1	1
	1–550	0	12	0	12	0	12	0	12	0	12	0	12	6	0	3	3	1	11	0	12	2	0	1	1
	1–600	0	12	0	12	0	12	0	12	0	12	0	12	6	0	4	2	0	12	0	12	2	0	1	1
		PHENOL CONTROLS REPORTED OFFICIAL METHOD																					
		TIME IN MIN.																					
		5	10	15		5	10	15		5	10	15		5	10	15		5	10	15		5	10	15	
	1–80	+	+	−		+	−	−		+	−	−		+	−	−		−	−	−		+	−	−	
	1–90	+	+	+		+	+	−		+	+	+		+	+	−		+	−	−		+	+	−	
M. pyogenes var *aureus*		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE		F.D.A. BROTH		THIOGLYCOLLATE	
	1–140	11	1	10	2	6	6	5	7	11	1	12	0	6	0	6	0	12	0	12	0	2	0	2	0
	1–160	3	9	4	8	2	10	0	12	8	4	8	4	6	0	6	0	12	0	12	0	2	0	1	1
	1–180	0	12	0	12	0	12	0	12	1	11	1	11	6	0	6	0	12	0	12	0	1	1	1	1
	1–200	0	12	0	12	0	12	0	12	0	12	0	12	2	4	1	5	8	4	8	4	2	0	2	0
	1–250	0	12	0	12	0	12	0	12	0	12	0	12	3	3	1	5	0	12	0	12	2	0	2	0
		PHENOL CONTROLS REPORTED OFFICIAL METHOD																					
		TIME IN MIN.																					
		5	10	15		5	10	15		5	10	15		5	10	15		5	10	15		5	10	15	
	1–60	+	−	−		+	−	−		+	−	−		+	−	−		+	−	−		+	−	−	
	1–70	+	+	+		+	+	+		+	+	+		+	+	−		+	+	−		+	−	−	

The committee in interpreting these results stated that as a whole there seemed to be no difference in the test data when the thioglycollate broth was used for subculturing and where Nutrient broth was used. It should be noted that laboratories 1, 2, 3, and 5 secured results which were in fairly good agreement by both procedures when *S. typhosa* was the test organism. The results from laboratories 4 and 6 did not conform, but there was no appreciable difference in the results secured by these 2 laboratories by either method used. It should be noted that laboratory 5 indicated slightly higher results than laboratories 1, 2, and 3 with both subculture media, but this laboratory used a culture with a phenol resistance of 1–90, whereas laboratories 1, 2, and 3 used cultures of higher resistance. When *M. pyogenes* var. *aureus* was used as the test organism fairly good agreement was secured between all 6 laboratories by both procedures. Laboratories 1, 2, and 4 list data which would suggest that slightly lower results might be expected with both test organisms when thioglycollate medium was used for subculturing. This was not, however, borne out by the data submitted by laboratories 3 and 5. These two laboratories secured almost identical results with both subculture media.

As a further check on the suitability of Fluid Thioglycollate Medium for use as a subculture media in the official method another collaborative study was conducted to ascertain the effect of its use on the phenol coefficient when employed in subculturing in both the phenol control and the unknown medicant.

The collaborators in this study were as follows:

John Bogusky, Insecticide Division, Livestock Branch, Production and Marketing Administration, USDA, Beltsville, Maryland.
C. M. Brewer, Microbiological Division, Food and Drug Administration, Washington, D. C.
Michael J. Pelczar, Bacteriology Department, University of Maryland.

In this study replicates of 2 disinfectants were forwarded by Mr. Bogusky to the collaborators with instructions for testing by both the official method, and the official method modified to use the thioglycollate medium for subculture of both the phenol control and the experimental samples. Experiemental sample No. 1 was a commercial brand of disinfectant containing cresylic acid, iso-propyl alcohol, dodecyl benzene monosulfonate, and chloro-2 phenyl phenol, as active ingredients. Experimental sample No. 2 was an aliquot of this same product in which approximately 0.17 per cent of phenyl mercury acetate was incorporated. This represents approximately 0.1 per cent of metallic mercury. These 2 samples cannot be classed as representative of the most commonly distributed cresylic acid disinfectants; but they are representative of the types that most frequently cause trouble in testing. Both contain a synthetic anionic surface active salt and one also contains an organic mercurial of

high bacteriostatic potency. Tests were run against both *S. typhosa* and *M. pyogenes* var. *aureus*. The results are given in Table 2.

The results given in Table 2 show about the same degree of conformance between the results in the 3 laboratories when nutrient broth was used for subculturing and when Fluid Thioglycollate Medium was used. With sample 1 the results were identical when *M. pyogenes* var. *aureus* was used as the test organism. Two of the 3 laboratories reported identical

TABLE 2.—*Phenol coefficient values found on phenolic disinfectants in second collaborative study comparing A.O.A.C. method and A.O.A.C. method modified in that fluid thioglycollate medium was used for subculturing in lieu of Nutrient broth*

TEST ORG.	SAMPLE NO.	COLLABORATING LABORATORIES BY NUMBER					
		1		2		3	
		NUTRIENT BROTH	THIOGLYCOLLATE	NUTRIENT BROTH	THIOGLYCOLLATE	NUTRIENT BROTH	THIOGLYCOLLATE
S. typhosa	1	5.5	5.5	5.0	5.0	5.0	5.0
	2	7.9	7.4	8.9	5.6	8.2	5.6
M. pyogenes var. *aureus*	1	5.0	5.0	5.0	5.0	5.0	5.0
	2	6.5	4.1	6.9	5.3	6.6	5.8

results for *S. typhosa*, but 1 laboratory secured a slightly higher coefficient. With sample 2 all laboratories secured higher results when Nutrient broth was used than when Fluid Thioglycollate Medium was employed. This is, of course, an indication of the efficiency of the thioglycollate medium in overcoming the bacteriostatic effect of the mercury salt present. The coefficient of sample 2 was found to be higher than sample 1 in all but one instance even when Fluid Thioglycollate Medium was used in subculture, indicating that the addition of 0.17 per cent phenyl mercury acetate to formula 1 did increase germicidal activity.

Letheen Broth was first proposed as a culture medium by Quisno, Gibby, and Foter (*Amer. J. Pharm.*, **118**, 320–323, 1946). It was developed specificially for evaluating the germicidal potency of quaternary ammonium salts and was reported by the above authors to be capable of neutralizing the activity of the largest amount of germicide which would be transferred into subcultures under the conditions encountered in the phenol coefficient procedure. The neutralizing agent appears to be lecithin and the polyoxyethylene derivative of sorbitan monooleate acts as a solubilizing agent for this material, providing a clear medium. Subsequently, Warner, Pelczar, and Stuart (*This Journal*, **32**, 401–408, 1949) observed a direct quantitative relationship between the neutralizig effect of lecithin and quaternary ammonium salts which would tend to bear out

the contention that this medium was capable of neutralizing any bacteriostatic effects by these germicides which would normally be encountered in subculture tubes using the phenol coefficient procedure. The neutralizing value of the lecithin-polyoxyethylene derivative of sorbitan monooleate combination used in this medium for quaternary ammonium disinfectants was then carefully investigated by Weber and Black (*J. Bact.*, **54,** 44, 1947), (*Soap Sanit. Chemicals,* **24,** Sept., 1948). They reported that a ratio of 100 parts of lecithin to 1 part of quaternary would effectively neutralize bacteriostatic effects with species of bacteria normally used in laboratory test procedures, although higher ratios were necessary with some more sensitive species. As a result of this work, a combination of these chemicals was adopted for use in the swab-rinse plate count technique for evaluating the sanitizing efficiency of quaternary ammonium disinfectants for dishes, glasses, and eating utensils by Public Health Officials.

In a collaborative study conducted by the Disinfectant Scientific Committee of the National Association of Insecticide and Disinfectant Manufacturers in 1946, some of the collaborating laboratories reported results of tests on quaternary ammonium germicides made by both the official method and the official method modified in that Letheen broth was used to replace the F.D.A. or Nutrient broth in subculturing.

While the primary objective in this study was not a comparison of results by the official method using Nutrient broth and Letheen broth for subculturing, a number of collaborators on their own initiative submitted data which when combined provided the opportunity for making a general comparison of this type.

The collaborators submitting results used for this purpose were:

Jack Varley and Miss H. T. Schmitt, James Varley & Sons, Inc., St. Louis, Missouri.

C. A. Lawrence, Winthrop Chemical Company, Inc., Rensselaer, N. Y.

E. G. Klarmann and Mrs. E. S. Wright, Lehn & Fink, Bloomfield, New Jersey.

George F. Reddish and Robert G. Sanders, Lambert Pharmacal Company, St. Louis, Missouri.

L. S. Stuart and J. L. Friedl, Insecticide Division, Livestock Branch, Production and Marketing Administration, USDA, Washington, D. C.

Replicate samples of three commercial 10% quaternary ammonium germicide concentrates were forwarded by the Referee to all collaborators.

The results recorded using the A.O.A.C. technique against *S. typhosa*, three laboratories reporting, and the results recorded by this same technique modified to use Letheen broth for subculturing, 4 laboratories reporting, are presented in Table 3 in work sheet form.

The results in Table 3 seem to indicate that use of Letheen broth tends to lower the result slightly and provides a somewhat more uniform result with respect to both the level of germicidal activity and "wild plus" and "skip" readings. With Sample 1, the apparent coefficients found by the 3

TABLE 3.—*Comparison of results secured in collaborative studies against S. typhosa by the A.O.A.C. method and the A.O.A.C. method modified in that Letheen broth was used for subculturing in lieu of Nutrient broth*

DISINFECTANT	DILUTION	LABORATORIES USING A.O.A.C. TECHNIQUE—NUTRIENT BROTH SUBCULTURE									LABORATORIES USING A.O.A.C. TECHNIQUE—LETHEEN BROTH SUBCULTURE											
		1			2			3			1			2			3			4		
		5′	10′	15′	5′	10′	15′	5′	10′	15′	5′	10′	15′	5′	10′	15′	5′	10′	15′	5′	10′	15′
Phenol	1–80	−	−	−	−	−	−	+	−	−	+	−	−	−	−	−						
	1–90	+	−	−	+	−	−	+	−	−	+	+	+	−	−	−	+	−	−	+	−	−
	1–100	+	+	−	+	+	+	+	+	+				+	+	−	+	+	−	+	+	+
Q. A. Sample 1	1–600																−	−	−	−	−	−
	1–800	−	−	−							+	−	−				−	−	−	−	−	−
	1–1000	−	−	−	−	−	−				+	−	−				−	−	−	−	−	−
	1–1200				−	−	−	−	−	−	+	−	−				−	−	−	−	−	−
	1–1500	+	+	+	+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	+	−	−
	1–1800				+	−	−	+	−	−	+	+	+	−	−	−	−	−	−	+	−	−
	1–2000	+	−	−	−	−	+	+	−	−	+	−	−	−	−	−	−	−	−	+	−	−
	1–2400	+	+	+	+	−	−	+	−	−				+	+	+	−	−	−	+	+	+
	1–2700				+	+	−	+	−	−				+	+	+	−	−	−	+	+	+
	1–3000							+	+	+												
Q. A. Sample 2	1–600	−	−	−													+	−	−	−	−	−
	1–800	+	−	−										+	−	−	+	−	−	+	−	−
	1–1000	+	+	+	−	−	−				+	+	+	+	+	+	+	−	−	+	−	−
	1–1200				−	−	−	+	−	−	+	+	+	+	+	+	−	−	−	+	+	−
	1–1500	+	−	−	+	−	−	+	−	−	+	+	+	+	+	−	+	+	−	+	+	+
	1–1800				+	−	−	+	−	−	+	+	+	+	+	+				+	+	+
	1–2000	+	+	+	+	+	−	+	+	−	+	+	+	+	+	+						
	1–2400	+	+	+	+	+	−	+	+	+												
	1–2700				+	+	+	+	+	+												
	1–3000																					
Q. A. Sample 3	1–600	−	−	−																		
	1–800	+	+	+										−	−	−	−	−	−	−	−	−
	1–1000	−	−	−	−	−	−				+	−	−	−	−	−	−	−	−	−	−	−
	1–1200				+	−	−	−	−	−	+	−	−	−	−	−	−	−	−	−	−	−
	1–1500	+	+	−	+	−	−	+	−	−	+	+	+	−	−	−	−	−	−	−	−	−
	1–1800				+	−	+	+	−	−	+	+	+	+	+	−	+	−	−	+	−	−
	1–2000	+	+	+	+	−	−	+	+	−	+	+	+	+	+	+	+	+	−	+	+	+
	1–2400	+	+	+	+	+	+	+	−	+										+	+	+
	1–2700							+	+	−												
	1–3000	+	+	+																		

laboratories were 13.9, 26.6, and 30.0 by the A.O.A.C. method. Where Letheen broth was used three laboratories found probable coefficients of 16.6, 21.5, 22.2, while the fourth found no definite end point. With sample 2, the calculated coefficients by the A.O.A.C. method were 8.8, 20.0 and 20.0, whereas values of 8.8, 16.6, and 13.3 were indicated when Letheen broth was used. With sample 3, coefficients of 7.7, 22.2, and 26.6 were indicated by the A.O.A.C. method, while values of 13.3, 15.7, 20.0, and 20.0 were indicated when the method was modified to employ Letheen broth for subculturing.

In this study, the correlation between different laboratories cannot be reported as satisfactory by either method, but it appeared to be somewhat better when Letheen broth was employed instead of Nutrient broth for subculturing.

As a result of the data shown in Table 3 and other collaborative test data submitted and reported (*Soap Sanit. Chemicals*, **23**, 135, 1947), the Insecticide Division, U. S. Department of Agriculture, announced that Letheen broth would be employed for subculturing in phenol coefficient tests on quaternary ammonium germicides.

It should be noted here that three of the collaborating laboratories made comment that use of Letheen broth for subculturing aided in eliminating "wild plus" and "skip" readings, while one found it unsatisfactory as a subculture medium.

Another collaborative study was undertaken to determine comparatively the results which would be obtained by the official method and the official method modified in that Letheen broth was substituted for Nutrient broth in subculturing.

The collaborators were again:

John Bogusky, Insecticide Division, Livestock Branch, Production and Marketing Administration, USDA, Beltsville, Maryland.
Michael J. Pelczar, Bacteriology Department, University of Maryland.
C. M. Brewer, Microbiological Division, Food and Drug Administration, Washington, D. C.

Two commercial disinfectants were selected for this study. Sample 1 was an ordinary pine oil disinfectant containing less than 10% water, with pine oil and soap as active ingredients. Sample 2 was a pine-type disinfectant containing pine oil and a quaternary ammonium salt as active ingredients and 44% of water. Both gave stable emulsions in water.

Replicate samples were prepared by Mr. Bogusky for the collaborative tests. The results are recorded in Table 4.

The data in Table 4 shows that in all but one instance each laboratory secured identical results with the pine oil disinfectant when Nutrient broth and Letheen broth were used for subculturing. Laboratory 3 found a slightly higher result with Nutrient broth than with Letheen broth when *S. typhosa* was the test organism. All 3 laboratories found this product to

be ineffective against *M. pyogenes* var. *aureus*. Laboratory 1 found higher results for this product than laboratories 2 and 3, but this variation cannot be attributed to the subculture medium. With the pine-type disinfectant containing a quaternary ammonium salt, all 3 laboratories secured coefficients ranging from approximately 25 to 33 per cent lower when Letheen broth was used in lieu of Nutrient broth for subculturing. The results reported by laboratories 2 and 3 were in very close agreement with both test organisms and the 2 procedures employed. Laboratory 1 secured somewhat higher results with *S. typhosa* and somewhat lower results

TABLE 4.—*Phenol coefficient values secured on commercial samples of pine oil and pine type disinfectants by the A.O.A.C. method and the method modified in that Letheen broth was used for subculturing in lieu of Nutrient broth*

TEST ORG.	DISINFECTANT	COLLABORATING LABORATORIES					
		1		2		3	
		NUTRIENT BROTH	LETHEEN BROTH	NUTRIENT BROTH	LETHEEN BROTH	NUTRIENT BROTH	LETHEEN BROTH
S. typhosa	Sample 1 Pine Oil	5.0	5.0	4.4	4.4	4.7	4.4
	Sample 2 Pine Type	11.1	8.3	8.3	5.5	8.3	5.5
M. pyogenes var. *aureus*	Sample 1 Pine Oil*	0	0	0	0	0	0
	Sample 2 Pine Type	16.6	12.5	23.0	15.0	23.0	18.0

* All three laboratories found that a 1–10 dilution failed to kill the organism.

with *M. pyogenes* var. *aureus*. These variations appear to be due to factors other than the subculture medium.

Inasmuch as the phenol coefficient technique is widely used as a method for evaluating the germicidal activity of preparations and mixtures which may not act in the same manner as phenol, and where the results, if expressed as a calculated coefficient value, may be misleading, it seems essential to make certain other changes in wording to establish more firmly the official nature of the testing procedure itself irrespective of the method of recording, or the interpretation given to results. Thus, the following changes would seem appropriate.

The parenthetical statement preceding the text of the method (p. 86) and following the title should be changed to read:

"(Technique applicable to testing disinfectants miscible with H_2O which act against bacteria in a manner somewhat comparable to phenol and which do not exert bacteriostatic effects that cannot be neutralized by one of the 3 subculture media specified.)"

The title of Fig. 11 (p. 88) should be revised to read: "Transfer Loop and Manner of Using in Phenol Coefficient Technique."

Under **6.156,** Calculation, the first sentence should be revised to read as follows:

"Express results in terms of the phenol coefficient number or the highest dilution killing the test organism in 10 min., but not in 5 min. whichever most accurately reflects the germicidal value of the disinfectant." The next sentence should then read: "The phenol coefficient is a number obtained by dividing numerical value of greatest diln. (denominator of fraction expressing dilution) of the disinfectant capable of killing *S. typhosa* in 10 min. but not in 5 min. by the greatest dilution of phenol showing same results." The "Note," under **6.156,** should then be changed to read as follows: "NOTE.—The commonly accepted criterion that disinfectants for general use be employed at a diln. equivalent in germicidal efficiency to 5% phenol against *S. typhosa* (that is, 20 × the *S. typhosa* coefficient) allows a reasonable margin of safety for disinfection of infective agents likely to be the object of general disinfection. With products where this does not hold true such dilutions should not be used."

The changes outlined above are all that can be justified at this time.

Corper and Cohn (*J. Bact.*, **35,** 223, 1938) have pointed out that composition of the test culture medium probably plays the predominant role in determining both thermal and chemical resistance of bacteria; and, variations in results between laboratories due to variations in lots of commercial peptone used in the present Nutrient broth were initially reported by Brewer (*Am. J. Pub. Health,* **33,** 261, 1943). Differences in results due to this cause provide a source of constant difficulty. Thus, experimental collaborative work should be continued in an effort to find a more satisfactory chemically defined medium for maintaining and propagating test cultures than Nutrient broth. Some progress has been made in the development of a medium of this kind, Wolf (*J. Bac.*, **49,** 463, 1945), but further fundamental and collaborative study on this point will be necessary before any change in the present method can be considered.

Considerable interest has been expressed in the tentative Fungicidal Test (47) which appears on pages 90 and 91 of the sixth edition. It is proposed that this method be now designated as First Action. The following editorial changes are also proposed. Under **6.158,** the last sentence should be changed to read: "Strain No. 640, American Type Culture Collection, is suitable." A parenthetical section should be inserted immediately beneath the title to read: "(Applicable for use with H_2O miscible type fungicides used to disinfect inanimate objects)." Under **6.161,** the third sentence should be changed to read: "Free conidia from mycelium by shaking mixture with glass beads, filter thru sterile absorbent cotton to remove hyphal elements, and dilute with physiological NaCl soln so that the test suspension will contain 5 million conidia/ml." The concluding sentence of the section would then read, "Estimate densities of conidia in suspension microscopically using haemacytometer."

RECOMMENDATIONS*

(1) It is recommended that the "Phenol Coefficient (44)—official test" **(6.153–6.157)** be revised as follows (first action):

(a) Change the names and abbreviations for the test organisms *Eberthella typhosa* and *Staphylococcus aureus* to conform with the nomenclature used for these bacteria in the 1948, sixth edition, of Bergey's Manual.

(b) Provide a more complete identification of the test organisms according to the strain numbers recorded in the American Type Culture Collection.

(c) Make agitation of medicant tubes and subculture tubes mandatory at specified times during the test.

(d) Stipulate a sterilization time for media at 15 lbs. steam pressure of 20 min. instead of the present 40 min. period.

(e) List Nutrient broth, Fluid Thioglycollate Medium U.S.P. XIII, and Letheen broth as alternate subculture media for use, dependent upon whichever one gives the lowest result.

(f) Allow for use of platinum alloy transfer loops in addition to platinum loops for making culture transfers.

(2) That an Associate Referee be appointed to initiate studies directed toward the development of a more precise chemically-defined medium for maintaining and propagating test cultures used in the phenol coefficient method.

(3) It is also recommended that the "Fungicidal Test" **(6.158–6.162)** be made first action.

* For report of Subcommittee A and action of the Association see *This Journal*, 33, 41 (1950).

WEDNESDAY—MORNING SESSION

REPORT ON PLANTS

By ELROY J. MILLER, (Michigan Agricultural Experiment Station, East Lansing, Michigan) *Referee*

The Associate Referees on methods for analyzing Plant Materials have made substantial progress in their respective studies during the past year.

The following reports were prepared and submitted for presentation at the meeting of the Association this fall:

Report on Carotene and Zinc in Plant Tissue by Erwin J. Benne, Associate Referee, and Betty R. Johnston.

Report on Copper and Cobalt in Plants by Kenneth C. Beeson, Associate Referee, and Richard L. Gregory.

Report on Sodium in Plants by Ray L. Shirley, Associate Referee, and Erwin J. Benne.

Report on Sugars in Plants by Kenneth T. Williams, Associate Referee, Arthur Bevenue, and Earl F. Potter.

Report on Starch in Plants by Carroll L. Hoffpauir, Associate Referee.

RECOMMENDATIONS*

It is recommended—

(1) That the Associate Referees listed on page 4, *This Journal* **32,** (1949) continue the study of their respective assignments.

(2) That the following recommendations by the Associate Referees be accepted.

Carotene:

(*1*) That methods **12.75** and **12.76** be adopted, first action.

(*2*) That (4) under **12.77**, Supplementary Information, be changed to read as follows: "The Ba $(OH)_2$ may be added in a saturated aqueous soln, which is used to bring the acetone to 85% by volume. The effectiveness of this solution for removing chlorophyll is greatly increased by adding 10 gms. of NaOH to each liter."

(*3*) That a study of the subject be continued.

Zinc:

(*1*) That the method and its divisions which are numbered **12.24–12.30,** inclusive, be adopted as first action.

(*2*) That the modification of the original method as published in *This Journal,* **32,** 276 (1949), be adopted as an alternative procedure first action.

(*3*) That a study of the subject be continued.

* For report of Subcommittee A and action of the Association, see *This Journal,* **33,** 41 (1950).

Copper and Cobalt:

(*1*) That the Associate Referee determine the reasons for sample variation with respect to cobalt determination and correct that difficulty.

(*2*) That comparative analyses using both the nitrosocresol method and the nitroso-R-salt method be studied collaboratively with the objective of selecting the method most suited to routine work.

(*3*) That collaborative work on the carbamate method for copper be continued and extended.

Sodium:

(*1*) That the magnesium uranyl acetate method **(12.20–12.21)** be adopted, first action.

(*2*) That the study of methods for determining sodium in plant materials be continued.

Sugars:

(*1*) That the Hassid ferricyanide method for sugar analysis be further investigated in preparation for collaborative study.

(*2*) That any collaborative study of the A.O.A.C. micro method and Somogyi modified micro method be comprehensive and that special emphasis be given to small amounts (0.10 to 0.50 mg) of sugar.

(*3*) That various ion-exchange resins be studied to determine their value in clarifying plant extracts for sugar analysis.

Starch:

(*1*) That a collaborative study of the method described for the determination of starch in plant tissues be conducted.

(3) That the following methods be adopted, first action:

12.3, Moisture
12.4, Ash
12.11, Aluminum
12.18, Potassium
12.22, 12.23, Copper
12.31–12.32, Arsenic
12.47, Iodine
12.48, Selenium
12.50, Reducing Sugars
12.51–12.52, Reducing Sugars
12.53 A & B, Sucrose
12.54, Ether Extract
12.55, Crude Fiber
12.56, Total Nitrogen
12.57, Organic and Ammoniacal Nitrogen
12.64–12.67, Lignin
12.78, Boron

(4) That the following methods be deleted:

12.58–12.59, Ammonia in Tobacco

12.60–12.61, Free Nicotine in Tobacco
12.62–12.63, Nitrate Nitrogen in Tobacco

(5) It is also recommended that the 72% sulfuric acid method, *This Journal,* **32,** 287–291 (1949), be adopted as an alternative method first action.

REPORT ON SODIUM IN PLANTS

By RAY L. SHIRLEY,[1] *Associate Referee,* and ERWIN J. BENNE
(Michigan Agricultural Experiment Station, East Lansing, Michigan)

Last year the authors reported the results of a study of the tentative A.O.A.C. magnesium uranyl acetate method for determining sodium in plants.[2] At the meeting of the Association and subsequently, numerous investigators have expressed interest in knowing how values for sodium by this method would agree with those obtained for the same plant materials by use of the flame photometer. Hence, it seemed of value to obtain information on this point through a collaborative study of the subject.

Accordingly, the authors contacted a number of investigators known to have flame photometers and several expressed willingness to use their instruments for analyzing collaborative samples of plant materials for their sodium contents. Each of them was furnished samples of four different kinds of plant materials which the Associate Referee had analyzed for sodium by use of the tentative A.O.A.C. procedure with the results given below:

Kind of plant material	*Na, per cent*
Alfalfa hay	0.040
Celery plants	0.953
Corn grain	0.269
Soybean oil meal	0.012

RESULTS AND COMMENTS OF COLLABORATORS

Only three collaborators had reported by the early date at which this report had to be submitted. Their results and comments follow:

Dr. O. J. Attoe and Mr. Wang, Department of Soils, University of Wisconsin, Madison, Wis.—The collaborative samples were analyzed for sodium by use of a Perkin-Elmer Flame Photometer, Model 18, with the results given in the table below.

It will be noticed that the data given for determinations made on extracts of the tissue are nearly identical with those made on extracts of the ash. Also, results are given in which a correction is made for the interference that calcium has on the determination of sodium with this in-

[1] Present address: Florida Agricultural Experiment Station, University of Florida, Gainesville, Florida.
[2] *This Journal,* 32, 280 (1949).

KIND OF TISSUE	% Na FOUND			
	TISSUE EXTRACTED WITHOUT PREVIOUS ASHING		TISSUE ASHED BEFORE EXTRACTION	
	NOT CORRECTED FOR Ca INTERFERENCE	CORRECTED FOR Ca INTERFERENCE	NOT CORRECTED FOR Ca INTERFERENCE	CORRECTED FOR Ca INTERFERENCE
Alfalfa hay	0.056	0.043	0.057	0.044
Celery plants	1.04	1.03	1.00	0.99
Corn grain	0.33	0.33	0.31	0.31
Soybean oil meal	0.024	0.024	0.024	0.024

strument. This correction is usually very small or negligible, except where the content of sodium is very low and that for calcium is rather high, such as in the case of the alfalfa hay sample.

The advantages of making the determinations without ashing the tissue are quite obvious. This technique seems to work very well on many kinds of plant tissue. It is possible that it might not work well on tissue that is quite oily or waxy.

The following procedure was used in making the sodium analyses:

REAGENTS

Stock solution of ammonium and magnesium acetates for preparation of extracting and calibrating solutions.—This solution is 4 *N* with respect to ammonium acetate and 0.4 *N* with respect to magnesium acetate. It is prepared by diluting 228 ml of glacial acetic acid with about 300 ml of water, and then adding with agitation 270 ml of concentrated ammonium hydroxide. To this is added 42.8 grams of $Mg(C_2H_3O_2)_2 \cdot 4H_2O$. The soln is adjusted to *p*H 6.9 with ammonia or acetic acid, as required, and finally diluted to 1 liter. The addition of an excess of ammonia should be avoided as far as possible because it necessitates the addition of acetic acid which would alter normality relationships.

Extracting solution.—The extracting soln is 2 *N* with respect to ammonium acetate and 0.2 *N* with respect to magnesium acetate. It is prepared by diluting a portion of the above stock soln with an equal volume of distilled water.

Standard stock solutions of KCl and NaCl.—Dissolve 1.907 grams of KCl in water and dilute to 1 liter. Similarly, dissolve 2.541 grams of NaCl and make up to 1 liter of soln. These now contain 1000 p.p.m. of K and Na, respectively.

Standard calibrating solutions of KCl and NaCl.—Standard calibrating solns containing 10, 20, 50, and 100 p.p.m. of K and Na are sufficient in most cases. To prepare 500 ml of each of these solns the following volumes of stock solns are taken and diluted to 500 ml.

CONTENT OF K OR Na DESIRED IN CALIBRATING SOLUTION	VOLUMES OF STOCK SOLUTIONS NEEDED	
	OF SOLUTION CONTAINING 1000 P.P.M. K OR Na	OF SOLUTION 4 *N* AS REGARDS $NH_4C_2H_3O_2$ AND 0.4 *N* AS REGARDS $Mg(C_2H_3O_2)_2$
p.p.m.	*ml*	*ml*
10	5	250
20	10	250
50	25	250
100	50	250

EXTRACTION AND DETERMINATION OF POTASSIUM AND SODIUM

Extraction.—A sample, usually about 0.5 gm, of plant tissue ground to pass a sieve with 1 mm openings is placed in a 500 ml (or other suitable size) flask and 100 ml of extracting soln added. The flask is stoppered and shaken mechanically for one hour or intermittently by hand over the same period of time. The suspension is then filtered, using Whatman No. 2 or similar grade of filter paper. The extract is now ready for testing with the flame photometer.

In the case of grain, the flask containing the sample with extracting soln is stoppered and allowed to stand for 1 to 2 hours at about 50°C. before shaking and filtering.

If it is desired to first destroy the organic matter before soln of the potassium and sodium, the sample is placed in a 250-ml beaker and 10 ml of nitric acid are added. The beaker is covered, placed on the hot plate, and allowed to heat until the tissue is quite thoroly decomposed. It is then removed and 5 ml of dilute nitric acid (1 to 1) are added, followed by the addition of 2 ml of 60% perchloric acid. The beaker is covered and again placed on the hot plate. When the last evidence of organic matter has disappeared, the cover is removed and the contents evaporated to dryness at a temperature just below the boiling point. The beaker is again removed and allowed to cool, when 100 ml of extracting soln are added and the residue policed and otherwise thoroly stirred so as to dissolve all of the soluble salts. The soln is filtered, using Whatman No. 2 or similar grade of filter paper. The extract is now ready for testing with the photometer.

Operation of flame photometer.—(Model 18, manufactured by the Perkin-Elmer Corporation.) The operation of the instrument depends upon the atomization of the test soln of potassium or sodium into the air supply of a Meker burner under controlled conditions, and a photoelectric measurement of the intensity of the potassium or sodium flame produced. The necessary steps in the operation of the instrument are the following:

(1) Fill the water cell at the top of the instrument to the line with distilled water. If allowed to run low, the photo tubes will be damaged.

(2) Turn range knobs (Nos. 1 and 3) and zero adjusting knobs (Nos. 4 and 5) to zero (counter-clockwise), and set ratio dial (No. 6) at zero.

(3) Turn on amplifier with switch, directly below ratio dial.

(4) Turn on the air to the instrument, and with the control knob (No. 7), adjust pressure to 20 pounds per square inch.

(5) Turn on gas, and light instruments burner and also burner used to burn excess gas from pressure stabilizer.

(6) Allow instrument to warm up for one-half hour or more and keep burner lighted during entire run of a series of determinations.

(7) Turn the element selector switch (No. 2) to either Na or K side, which ever is to be determined.

(8) With a ratio dial (No. 6) set on zero, place a 50 ml beaker containing about 20 ml of fresh or unused extracting soln into the test soln receptacle of the instrument so that the glass tube of the atomizer extends into the liquid. Set the meter at zero by turning the range control (No. 4 or 5). A slight tapping of the instrument facilitates this setting. Remove the beaker with soln.

(9) Turn the ratio dial to 100; now place a 50 ml beaker containing a standard soln, say 100 p.p.m. of K or Na, whichever is being determined, into the receptacle so that the glass tube of the atomizer extends into the liquid. Adjust the range control (No. 1 or 3) so that the meter reads zero. A *slight* tapping of the instrument facilitates this setting. Continue atomizing the soln and adjusting the range control until the reading remains practically constant. Remove the beaker with soln and

wash the tube with distilled water. *The instrument setting is now ready for determination of unknowns.*

(10) A 50 ml beaker containing about 20 ml of plant extract is similarly placed in the receptacle, and the pointer adjusted to zero by means of the ratio dial (No. 6). The reading of the ratio dial at this point is a measure of the concentration of K or Na in the plant extract in terms of the standard. The standard soln should be of such a concentration that readings taken from the dial do not fall below about 20. At least two separate readings should be taken for each sample. If these do not agree to within 2 or 3 per cent, a third and perhaps a fourth reading should be taken.

In case the concentration of K or Na in the plant extract is greater than that of the standard soln being used, it will be necessary to use a more concentrated standard soln or to dilute the plant extract. Ordinarily, 100 p.p.m. is the maximum concentration of K or Na used in the standard soln. When the plant extract contains a greater concentration than this, it is usually desirable to dilute a portion of it with a known volume of extracting soln.

Calculation.—For use in translating dial readings to p.p.m. of K or Na in soln, a graph is made by plotting dial readings obtained with a series of standard solns against respective p.p.m. of K or Na. The p.p.m. in the unknown are then read from the graph. Per cent K or Na in the plant tissue may be calculated by means of the following formula:

$$\% \text{ K or Na} = \frac{\text{ml. of extract} \times \text{p.p.m. K or Na in extract} \times .0001}{\text{weight of sample}}.$$

Errors involved in this method of determining potassium and sodium are usually less than 3% of actual amounts.

Precautions.—Use only filtered test solns because atomizer jet easily becomes clogged if cloudy solns are used. It is necessary to re-standardize the instrument every 3 or 4 min. with the extracting soln and a standard soln of K or Na. Extra care should be taken to keep the standard solns free from contamination or dilution. Check frequently to see that the water cell at the top of the instrument is filled to the line with distilled water.

Firman E. Bear and Stephen J. Toth, N. J. Agricultural Experiment Station, Department of Soils, Rutgers University, New Brunswick, N. J.— The collaborative samples were analyzed for their Na content and the results are given in the following table. The content of Ca and K in the tissues are also reported in the table.

	PER CENT								
	Ca			Na			K		
	NO. 1	NO. 2	AVERAGE	NO. 1	NO. 2	AVERAGE	NO. 1	NO. 2	AVERAGE
Afalfa hay	1.48	1.54	1.51	0.036	0.036	0.036	2.65	2.60	2.63
Celery plants	2.30	2.45	2.37	1.07	1.03	1.05	4.10	4.20	4.15
Corn grain	0.08	0.12	0.10	0.275	0.285	0.280	0.37	0.40	0.38
Soybean oil meal	0.26	0.28	0.27	0.017	0.020	0.018	2.17	2.15	2.16

The details of the procedure used in preparing the samples for analysis and the make of the flame photometer used are published in *Soil Science,*

66, 459 (1948). In general, the dry plant material was wet-ashed successively with nitric and perchloric acids, silica was removed by filtration, and the filtrate was made to a definite volume. Sodium was determined in aliquots of this solution by use of a Perkin-Elmer flame photometer, model 52A, using acetylene gas.

The values for Ca, K, and Na in the tissues represent duplicate wet digestions, and each recorded value is the mean of three readings on the flame photometer. For any value below 0.025 per cent Na, the estimation by the flame technique using acetylene gas is not too good. The reason for this is because of the activation of Ca ions in the flame which affect the Na values. It seems to us that under this condition, the substitution of the propane flame is more desirable. In our laboratories, however, we do not use the acetylene and propane burners interchangeably since the shifting from one to the other is too time consuming.

We would also like to point out that it is necessary to vary the size of the aliquots for the estimation of Ca, K, and Na contents of various plant tissues. Normally, this procedure is not necessary if the procedure is applied to a series of experimentally grown plants of the same species and variety.

With respect to the speed of the analysis by the flame techniques, it may be sufficient to say that the estimation of Ca, K, and Na in duplicate, on the four samples submitted, required only 3 hours from the time of weighing out the samples for wet ashing.

ADDENDUM

Alston W. Specht, U.S.D.A., Plant Industry Station, Beltsville, Md.— The Perkin-Elmer Model 52A flame photometer was used to analyze the collaborative samples with the following results.

NAME OF SAMPLE	% SODIUM (AIR DRY BASIS)	% SODIUM (OVEN DRY BASIS)
Alfalfa hay	.054	.052
Celery plants	1.08	1.22
Corn grain	.334	.398
Soybean oil meal	.022	.016

Sample and standard preparation.—For the determinations on the oven dry basis a portion of the samples were dried at 95°C. for 48 hours. One hundred mgm. each of air dried and oven dried material were slowly ashed in Vycor dishes by gradually advancing the furnace temperature from 200°C. to a maximum of 500°C. The ash was digested in 5 ml of 1:100 HCl soln for several hours and then diluted to 25 ml with the addition of 1:1000 HCl. Further dilution of the celery plant and corn grain solns was necessary to obtain a concentration of sodium similar to that of the other materials. The determination of sodium in all samples was made by using a set of standard solns covering a concentration range of zero to ten p.p.m. These standards were prepared using 1:1000 HCl and C.P. NaCl. All solns contained 100 p.p.m. of lithium for the internal standard element.

Comments.—Sodium free glass ware or platinum dishes should be used for ashing plant materials in preparation for sodium analysis when the sodium concentration in the solution to be analyzed will be in the order of one to ten p.p.m.

Examination of the data shows that the differences between the results obtained with the flame photometer and the Magnesium Uranyl Acetate procedure are progressively less as the quantity of sodium in the plant material increases.

DISCUSSION

It will be noted from the foregoing tables that the values for sodium in the collaborative samples obtained by the flame photometer agreed quite well with those determined by the Tentative A.O.A.C. procedure. Since a considerable amount of time can be saved by using the flame photometer, its use should prove advantageous in determining sodium for some purposes. Consequently, the authors feel that the study should be continued and extended.

RECOMMENDATIONS*

The Associate Referee recommends—

(1) That the tentative magnesium uranyl acetate method be made first action.

(2) That the study of methods for determining sodium in plant materials be continued.

REPORT ON STARCH IN PLANTS

By CARROLL L. HOFFPAUIR (Southern Regional Research Laboratory,[1] New Orleans, Louisiana), *Associate Referee*

A method for the determination of small amounts of starch in plants was recently published by Pucher, Leavenworth, and Vickery (1). This method involves extraction of starch from plant tissues with cold perchloric acid, precipitation of the starch with iodine, regeneration of the starch with alcoholic sodium hydroxide, hydrolysis to glucose and determination of reducing sugars by the Somogyi method (2). An investigation of the method was undertaken as a preliminary to collaborative work. Since there is widespread objection to the use of perchloric acid, based on the potential hazard involved, a modification of the Pucher, Leavenworth, and Vickery method, in which the starch is extended with boiling calcium chloride solution, as suggested by Sullivan (3) and others (4, 5), was also tried. Details of both the methods follow.

* For report of Subcommittee A and action of the Association see *This Journal*, 33, 41 (1950).

[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

REAGENTS

Calcium chloride soln.—Dissolve 2 parts of crystalline calcium chloride hexahydrate in 1 part of water. Adjust to a density of 1.30. Make very faintly pink to phenolphthalein with 0.1 *N* sodium hydroxide and filter. Add 6 ml of 0.8% acetic acid to each 100 ml of soln.

Perchloric acid.—72%, 11.2 to 11.7 *N* reagent grade.

Iodine-potassium iodide soln.—Grind 7.5 g of iodine and 7.5 g of potassium iodide with 150 ml of water. Dilute to 250 ml and filter.

Uranyl acetate.—5% aqueous soln.

Sodium chloride.—20% aqueous soln.

Alcoholic sodium chloride.—Mix 350 ml of ethanol, 80 ml of water, and 50 ml of 20% aqueous sodium chloride and dilute to 500 ml with water.

Alcoholic sodium hydroxide, 0.25 N.—Mix 350 ml of ethanol, 100 ml of water, and 25 ml of 5 *N* sodium hydroxide, and dilute to 500 ml with water.

Hydrochloric acid, 0.7 N.—Dilute 60 ml of concentrated hydrochloric acid to 1 liter with water.

Somogyi's phosphate sugar reagent.—Dissolve 56 g of anhydrous disodium hydrogen phosphate and 80 g of Rochelle salt in ca 1 liter of water, and add 200 ml of 1.00 *N* sodium hydroxide. Then add slowly 160 ml of 10% copper sulfate pentahydrate soln while stirring the soln. Dissolve 360 g of anhydrous sodium sulfate in the soln, transfer to a 2-liter volumetric flask, and add exactly 200 ml of 0.1 *N* potassium iodate soln (3.5760 g per liter) prepared with quantitative accuracy. Dilute the mixture to volume, mix well, allow to stand for several days, and filter thru dry paper into a dry flask, discarding the first 50 ml of filtrate. Store the reagent at 20°–25°C. It is 0.01 *N* with respect to iodate and 5.00 ml are equivalent to 10 ml of 0.005 *N* sodium thiosulfate. The effective range for the determination of sugar is from 0.05 to 1.0 mg of glucose in a 5-ml aliquot.

Sodium thiosulfate, 0.005 N.—Dissolve 2.73 g of sodium thiosulfate pentahydrate in water and dilute to 2 liters. Standardize the soln daily as follows: Add 1 ml of 2.5% potassium iodide and 3 ml of 1.5 *N* sulfuric acid to 5 ml of the Somogyi sugar reagent. Allow to stand for 5 min. and then titrate with the thiosulfate soln adding starch indicator when the titration is nearly completed.

Starch indicator.—Suspend 1 g of starch in cold water, pour the suspension into 200 ml of boiling water, and boil for a few min.

Potassium iodide.—2.5% soln stabilized with a little sodium carbonate.

PROCEDURE

Calcium chloride extraction.—Prepare sample as directed in method **12.2(b)** of the *Official and Tentative Methods of Analysis* (6). Dry and grind to smaller than 80 mesh. Weigh accurately 50–250 mg of dry powdered tissue into a 100-ml heavy pyrex centrifuge tube 30×170 mm. Add 5 ml of calcium chloride soln. Place the tube in an oil bath maintained at 120°C. and boil for 15 min. Stir the suspension frequently during the boiling period. Remove from the bath, cool, and dilute with 20 ml of water. Centrifuge for 10 min. at 2000 r.p.m. and decant the supernatant liquid into a 100-ml volumetric flask. Add a second 5 ml portion of calcium chloride soln to the residue, replace in the oil bath, and again boil for 15 min., with frequent stirring. Remove from the bath, cool, and transfer to the volumetric flask with distilled water. Add 3 ml of uranyl acetate soln to precipitate protein, make to volume with water, mix well, and centrifuge a portion. Pipette a 10-ml aliquot of the clear supernatant into a 25×150 mm pyrex test tube. Add 5 ml of 20% sodium chloride soln and 2 ml of iodine-potassium iodide reagent, and mix well. Allow to stand for at least 30 min., centrifuge, and decant the supernatant liquid.

Perchloric acid extraction.—Weigh accurately a 50–250 mg sample of dry powdered tissue into a pyrex test tube 150×25 mm. Add 200 mg of sharp sand and 4 ml of water. Heat the tube in a boiling water bath for 15 min. to gelatinize the starch. Cool to room temp. and place in a bath at 22°–25°C. Add 3 ml of perchloric acid rapidly with constant agitation. Grind the tissue against the lower wall of the tube with a stirring rod for a min. or so at a time. Repeat the grinding several times over a period of 15–20 min. Add 20 ml of water so as to wash the rod, mix well, and centrifuge. Decant the clear soln into a 50-ml volumetric flask. Reextract the residue with 4 ml of water and 3 ml of perchloric acid as before. Transfer to the volumetric flask with water, make to volume, mix well, and centrifuge a portion. Pipette a 10-ml aliquot of the clear soln into a 25×150 test tube. Add 5 ml of 20% sodium chloride soln and 2 ml of iodine-potassium iodide soln, mix well, and allow to stand for at least 30 min. Centrifuge and decant the supernatant liquid.

Determination of starch.—Wash the starch iodide precipitate obtained by either method described above by suspending it in 5 ml of alcoholic sodium chloride soln, centrifuge and decant the supernatant fluid. Add 2 ml of alcoholic sodium hydroxide soln to the packed precipitate. Gently shake and tap the tube until all the blue color is discharged. (A stirring rod must not be used and ample time must be allowed for the complex to decompose.) Centrifuge the liberated starch and wash with 5 ml of alcoholic sodium chloride as before.

Add 2 ml of 0.7 *N* hydrochloric acid to the precipitate. Loosely stopper the tube with a size 00 crucible, and heat in a boiling water bath for 2.5 hours. (The water bath should be provided with a cover with holes to accommodate the tubes. It is important that holes not occupied by tubes be covered.) Cool and transfer quantitatively to a 25-ml volumetric flask. Add a drop of 0.04% phenol red and neutralize with 1 *N* sodium hydroxide. Discharge the color with 0.1 *N* oxalic acid, make to volume and mix well. Transfer a 5 ml aliquot to a 25×200 mm pyrex tube, add exactly 5 ml of the Somogyi phosphate reagent, and stopper the tube with size 00 crucible. Heat together with several blanks containing 5 ml water and 5 ml of Somogyi reagent, in a vigorously boiling water bath for exactly 15 min. Remove the tube from the bath and cool to 25°–30°C. Add 1 ml of 2.5% potassium iodide down the wall of the tube without agitation and then add 3 ml of 1.5 *N* sulfuric acid rapidly with simultaneous agitation. After all the cuprous oxide has dissolved, titrate the soln with 0.005 *N* thiosulfate, adding starch indicator when the titration is nearly completed. Treat the blank solns similarly.

DISCUSSION OF RESULTS

Both the Pucher, Leavenworth, and Vickery method and the calcium chloride modification consist of several distinct operations, namely, the extraction of starch from the sample, the isolation of starch from the extract, the acid hydrolysis and the isolated starch to glucose, and the determination of glucose. These operations were each verified. In Table 1 are presented recovery data obtained by the Somogyi sugar method on aliquots of a solution of N.B.S. glucose. These recoveries average 100 7% indicating that the determination of glucose is satisfactory. To verify the acid hydrolysis step, carefully analyzed samples of sweetpotato and white potato starch, as well as N.B.S. glucose, were weighed on a microbalance into 25×150 mm pyrex test tubes, acid hydrolyzed as directed by the method, transferred to 25-ml volumetric flasks, neutralized, made to volume, and aliquots were used for the determination of reducing power. The values reported in Table 2 indicate satisfactory recovery.

TABLE 1.—*Recovery of dextrose using Somogyi reagent*

DEXTROSE ADDED	DEXTROSE FOUND	RECOVERY
mg	*mg*	*per cent*
0.1084	0.1090	100.6
0.2168	0.214	98.7
	0.218	100.6
0.3252	0.325	99.9
	0.329	101.2
0.4336	0.440	101.5
	0.437	100.8
0.5422	0.552	101.8
	0.552	101.8
Average		100.7

TABLE 2.—*Recovery of dextrose and starch after acid hydrolysis*

SAMPLE	ADDED	FOUND	RECOVERY
	mg	*mg*	*per cent*
Sweetpotato Starch	2.75	2.80	101.8
	2.77	2.82	101.8
White Potato Starch	2.46	2.47	100.4
	2.39	2.41	100.8
N.B.S. Dextrose	2.99	3.05	102.0
	3.51	3.51	100.0

Both the original and the modified extraction procedure were used on several samples reported on in the 1948 report of the Associate Referee on starch in plants (7). The cotton root bark sample is representative of woody tissues and contains considerable colored materials. The orange rind sample contains very little starch, and is rich in polysaccharides. Cottonseed meal is quite low in starch, high in protein, and contains appreciable raffinose. The Jerusalem artichoke sample has a high percentage of inulin but no starch. Sweetpotato starch was included as a check on recovery. Values obtained on these samples together with values previously reported (7) by a colorimetric method are given in Table 3. The colorimetric values were obtained using potato starch as a standard. The other values were calculated using the 0.90 factor for the conversion of starch to glucose. One ml. of 0.005 *N* thiosulfate was considered equivalent to 0.135 mg glucose as stated by Somogyi (2) and verified by data in Table 1.

Both the calcium chloride extraction and the perchloric acid extraction give values which are reasonable in view of what is known about the samples and which agree well with results obtained by the colorimetric

TABLE 3.—*Comparison of methods for determination of starch*
(Moisture-free basis)

SAMPLE	STARCH FOUND		
	COLORIMETRIC[1]	PERCHLORIC ACID EXTRACTION	CALCIUM CHLORIDE EXTRACTION
	per cent	*per cent*	*per cent*
Cotton root bark	4.6	4.54	4.40
		4.47	4.46
Orange rind	0.4	0.39	0.32
		0.39	0.36
Cottonseed meal	0.2	0.08	0.13
		0.08	0.13
Jerusalem artichokes	0.0	0.0	0.0
		0.0	0.0
Sweetpotato starch (99.3% starch) (by difference)		94.5	101.0
		93.5	101.2

[1] Potato starch standard.

method. The presence of pectin, inulin, raffinose, or protein does not interfere, and satisfactory recoveries were obtained on known amounts of starch. The extraction of plant materials such as wrinkled peas containing starch having unique properties has not been investigated. Such materials may require a modified extraction.

Since the final determination is based on the measurement of reducing power, the method, in contrast to colorimetric methods, is independent of the amylose-amylopectin ratio in the starch. It therefore meets the requirements of a satisfactory method for the determination of low percentages of starch in plant materials.

RECOMMENDATION*

With the development research completed, it is recommended that a collaborative study of the method described for the determination of starch in plant tissues be conducted.

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REPORT ON IODINE IN PLANTS

By FORREST G. HOUSTON and K. L. WOOD (*Associate Referee*)
(Kentucky Agricultural Experiment Station, Lexington, Kentucky)

A modification of the spectrophotometric method for the determination of iodine in plant material as devised by Gross, Wood, and McHargue (1) is briefly described in the following report. Details of the method and results obtained are presented elsewhere (2).

PROCEDURE

The air-dried sample is digested in the apparatus described by Matthews, Curtis, and Brode (3) with a mixture of chromium trioxide and concentrated sulfuric acid. The iodine is then reduced with phosphorus acid and isolated as hydrogen iodide by distillation into an alkaline absorbing solution. The iodides are oxidized to iodates by the permanganate technique. Free iodine is formed by the addition of potassium iodide, and the starch iodide chromogen developed in an ice bath. The total volume at this point is five milliliters. A Beckman model DU quartz spectrophotometer is used to measure the per cent transmission in a one centimeter cell and at a wave length of 575 millimicrons. Any other spectrophotometer can be used with the method and with slight changes many photoelectric colorimeters are suitable. The amount of iodine present is determined by reference to a standard curve prepared from standard iodine solutions. As little as 0.20 microgram of iodine can be determined with 10% accuracy, and larger amounts with greater accuracy. Consistent results are also obtained on soils and fertilizer materials with little change in procedure.

RECOMMENDATIONS*

It is recommended that the method be studied by others and collaborative work be undertaken for next year.

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REPORT ON SUGARS IN PLANTS

By KENNETH T. WILLIAMS, *Associate Referee*, ARTHUR BEVENUE, and EARL F. POTTER (Western Regional Research Laboratory,* Albany, Calif.)

During the past two years the Associate Referee and co-workers have studied and compared methods of analysis for sugars in plant materials. Three phases of the investigation are included in this report:

(1) Reducing sugars in a variety of vegetables were determined by the macro A.O.A.C. cuprous oxide method (1) and also by the micro ferricyanide method of Hassid (5, 6).

(2) A comparative study of the A.O.A.C. micro method (2) and the Somogyi modified micro method (10) was made on five different plant materials.

(3) A study of the clarification procedure was undertaken, substituting ion-exchange resins for several of the clarifying reagents more generally used.

When used in sugar analysis, ferricyanide reagents are more sensitive than copper reagents to substances of low reducing power. Therefore, higher sugar values may be obtained if a ferricyanide method is used (see 4, 7, 9, and others). However, this fact should not deter analysts from using this method, since it can be shown (Table 1) that with some plant

TABLE 1. *A comparison of values*[1] *obtained for reducing sugars from the edible portion of several raw vegetables by an official method and the Hassid method*

VEGETABLE	OFFICIAL METHOD (1)	HASSID METHOD (5, 6)
	per cent	*per cent*
Asparagus	1.97	2.05
Cabbage	3.31	3.50
Carrots	4.44	4.56
Cauliflower	1.34	1.33
Celery	0.93	0.89
Lettuce	1.80	1.69
Onion bulbs	4.68	4.58
Peas	0.14	0.16
Rhubarb	1.02	0.97
Zucchini squash	1.75	1.69

[1] The values given are averages of closely agreeing replicates.

materials it may be applicable (11). The ferricyanide method of Hassid has several advantages over many of the methods now in use. The titrating and indicator solutions are very stable, the ferrocyanide formed is not reoxidized by atmospheric oxygen, it requires but 2 mg, or less, of sugars, and because the reaction is stoichiometric, no tables are required

* Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.

to calculate the sugar values. The method is applicable if interfering non-sugar reducing substances can be removed (12) or if such substances are present in negligible quantities (8). (See Table 1 which contains reducing sugar values of a group of raw vegetables obtained by two methods of analysis, a copper method (1) and the Hassid method (5, 6), with sampled aliquots from the same clarified extract used for both methods.) The extracts were prepared as previously described (12), with neutral lead acetate, disodium phosphate, and Baker and Adamson Code No. 1551 decolorizing carbon (3) for clarification.

Somogyi's modified micro sugar method (10) was compared with the A.O.A.C. micro method (2) for the determination of reducing sugars in some plant materials as listed in Table 2. Four operators in this laboratory prepared their own analytical reagents and made the analyses independently for this comparison. The analyses were made from aliquots of a single clarified extract. Clarification of the extract consisted of treatment with neutral lead acetate, and with disodium phosphate to remove

TABLE 2.—*A comparison of values*[1] *obtained for reducing sugars from plant materials by the micro A.O.A.C. method and the Somogyi modified method*

PLANT MATERIAL	OPERATOR	MICRO A.O.A.C. METHOD (SOMOGYI) (2)		SOMOGYI MODIFIED METHOD (MICRO) (10)	
		per cent		*per cent*	
Alfalfa	1	(0.40, 0.40, 0.40, 0.40)	0.40	(0.40, 0.40, 0.40, 0.40)	0.40
	2	(0.40, 0.40, 0.40, 0.40)	0.40	(0.39, 0.39, 0.40, 0.39)	0.39
	3	(0.38, 0.38, 0.38, 0.38)	0.38	(0.44, 0.43, 0.43, 0.43)	0.43
	4	(0.38, 0.39, 0.39, 0.38)	0.39	(0.39, 0.39, 0.39, 0.39)	0.39
	Average		0.39		0.40
Cabbage	1	(2.93, 2.98, 2.96, 2.93)	2.95	(2.87, 2.87, 2.87, 2.87)	2.87
	2	(2.87, 2.87, 2.85, 2.72)	2.83	(2.89, 2.85, 2.85, 2.87)	2.87
	3	(2.86, 2.86, 2.84, 2.86)	2.86	(3.08, 3.09, 3.09, 3.06)	3.08
	4	(2.93, 2.91, 2.94, 2.96)	2.94	(2.87, 2.87, 2.89, 2.89)	2.88
	Average		2.90		2.93
Grass (Unidentified)	1	(0.25, 0.25, 0.25, 0.24)	0.25	(0.28, 0.28, 0.28, 0.28)	0.28
	2	(0.25, 0.26, 0.25, 0.25)	0.25	(0.27, 0.26, 0.27, 0.27)	0.27
	3	(0.24, 0.23, 0.23, 0.22)	0.23	(0.28, 0.28, 0.27, 0.28)	0.28
	4	(0.25, 0.24, 0.24, 0.25)	0.25	(0.27, 0.26, 0.26, 0.27)	0.27
	Average		0.25		0.28
Lettuce	1	(1.42, 1.43, 1.42, 1.42)	1.42	(1.38, 1.38, 1.38, 1.38)	1.38
	2	(1.42, 1.43, 1.43, 1.42)	1.43	(1.38, 1.36, 1.36, 1.36)	1.37
	3	(1.41, 1.39, 1.41, 1.38)	1.40	(1.48, 1.50, 1.49, 1.49)	1.49
	4	(1.42, 1.42, 1.42, 1.42)	1.42	(1.39, 1.36, 1.40, 1.39)	1.39
	Average		1.42		1.41
Potato (dehydrated)	1	(2.48, 2.48, 2.47, 2.48)	2.48	(2.55, 2.52, 2.53, 2.52)	2.53
	2	(2.47, 2.49, 2.49, 2.51)	2.49	(2.48, 2.45, 2.45, 2.45)	2.46
	3	(2.61, 2.61, 2.60, 2.51)	2.58	(2.75, 2.76, 2.74, 2.73)	2.75
	4	(2.65, 2.62, 2.65, 2.63)	2.64	(2.61, 2.56, 2.57, 2.61)	2.59
	Average		2.55		2.58

[1] The data are not selected as all of the values obtained by the operators are given in the table.

the excess lead. The data given in Table 2 do not demonstrate a superiority for either method. However, these data are too limited to support definite conclusions. The inclusion of sodium sulfate in the modified copper reagent to reduce reoxidation of the copper may be important in the determination of small amounts of sugar. Most certainly, the stability of the copper reagent is desirable.

The Associate Referee and co-workers have submitted a manuscript to the A.O.A.C. journal for publication, in which it has been shown that Amberlite IR 100 HAG and IR 4 BAG[1] ion-exchange resins are more effective than the neutral lead acetate-disodium phosphate-carbon treatment for the removal of non-sugar reducing substances from extracts of dehydrated white potatoes. In this work, four methods of sugar analysis (12) were used and compared. Concordant results were obtained when the extracts were clarified by the resins, whereas variable results were obtained by the lead-phosphate-carbon clarification.

A tentative program has been outlined by the Associate Referee to continue investigations on the use of ion-exchange resins in the preparation of plant extracts for sugar analysis, using plant crops directly from the field and also plant materials which have undergone some form of processing prior to use for human or animal consumption.

RECOMMENDATIONS*

It is recommended—

(1) That the Hassid ferricyanide method for sugar analysis be further investigated in preparation for collaborative study.

(2) That any collaborative study of the A.O.A.C. micro method and Somogyi modified micro method be comprehensive and that special emphasis be given to small amounts (0.10 to 0.50 mg.) of sugar.

(3) That various ion-exchange resins be studied to determine their value in clarifying plant extracts for sugar analysis.

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[1] The mention of commercial products does not imply that they are endorsed or recommended by the Department of Agriculture over others not mentioned.

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REPORT ON COPPER AND COBALT IN PLANTS

By KENNETH C. BEESON, *Associate Referee*, and RICHARD L. GREGORY (Plant, Soil, and Nutrition Laboratory, Agricultural Research Administration, U. S. Department of Agriculture, Ithaca, New York)

The carbamate method for copper and the nitroso-R-salt method for cobalt were examined and reported upon by the Associate Referee,[1] Lillian I. Butler, in 1942. At that time the Associate Referee recommended that work be continued on both methods and that they be submitted for collaborative study if found suitable. Subsequently, the copper method as recommended by the Associate Referee[2] was adopted as a tentative method, but no further study nor collaborative work has been reported.

Since this last report of the Associate Referee considerable improvement has been made in methods for both copper and cobalt with respect to speed, precision, and sensitivity. Cobalt methods are available, for example, that permit the determination of less than 0.01 gamma under proper conditions. This allows the use of relatively small samples as compared to the older nitroso-R-salt methods. In the opinion of the present Associate Referee, improvements have also been made in the carbamate methods for copper. Since no work had been done on either method for a number of years, and particularly since no collaborative work has ever been reported on these methods for plant tissue, it was decided to initiate studies on the Ellis and Thompson[3] method for cobalt and the sodium diethyldithiocarbamate method[4] as improved in this laboratory. Both methods depend also upon an improved ashing procedure.

COLLABORATORS

Since very few laboratories are doing work of this kind it has been difficult to secure collaborators. No volunteers were reported to the Associate Referee, but after extensive correspondence, seven persons agreed to try the methods. Of these, only three were able to obtain the necessary equipment or to complete the work in time for this report. For comparison, therefore, three persons including the authors in this laboratory analyzed the samples. It is recognized that this procedure does not necessarily permit any valid conclusion with respect to the usefulness of a method in the hands of different persons.

[1] *This Journal*, **25**, 568 (1942).
[2] *This Journal*, **25**, 567 (1942).
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The collaborators reporting are as follows:

(1) Professor Erwin J. Benne. Department of Agricultural Chemistry, Michigan State College, East Lansing, Michigan.
(2) Professor J. H. Mitchell. Department of Research Chemistry, South Carolina Experiment Station, Clemson, South Carolina.
(3) Dr. W. T. McGeorge. Department of Agricultural Chemistry and Soils, College of Agriculture, Tucson, Arizona.

In addition, the following members of the staff of this laboratory contributed data:

(4) Richard L. Gregory.
(5) Kenneth C. Beeson.
(6) Jean Dalrymple.
(7) Clayton J. Morris.

The methods submitted to the collaborators by the Associate Referee are as follows:

DETERMINATION OF COBALT AND COPPER IN PLANT TISSUE

A. REAGENTS FOR COBALT

(a) *Preparation of stock cupric O-nitrosocresol soln.*—Dissolve 6 g of hydroxylamine hydrochloride and 15 g of cupric chloride in 900 ml of distilled water and add 5 ml of *m*-cresol. These proportions are important, since they result in the optimum *p*H for the reaction. Add 15 ml of 30% hydrogen peroxide (Superoxol) with stirring. After standing 2 hrs at room temp, add 25 ml of concentrated hydrochloric acid and shake with 100–150 ml petroleum ether in a separatory funnel. Retain the petroleum ether extract. Repeat the extraction 3 or 4 times. Wash the yellow petroleum ether soln of the reagent several times with distilled water, after which shake successive 50–100-ml portions of 1% aqueous cupric acetate soln with the petroleum ether phase until it becomes colorless. Ca 300 ml of a deep red colored soln of the copper complex is obtained.

(b) *Sodium borate buffer.*—Dissolve 20 g of boric acid in 1 liter of redistilled water and add 50 ml of 1.0 *N* sodium hydroxide to bring the *p*H to 7.7–7.8. Equivalent volumes of sodium borate buffer and 0.01 *N* hydrochloric acid should give a soln of *p*H 7.0.

(c) *Sodium nitrosocresol soln.*—Add 10 ml of concentrated hydrochloric acid to 75 ml of stock cupric nitrosocresol soln in a separatory funnel and shake with 300–500 ml of petroleum ether. Remove the aqueous layer and shake the petroleum ether phase with two successive 100-ml portions of 0.01 *N* hydrochloric acid and then with two 100-ml portions of redistilled water. Add 25-ml portions of sodium borate buffer soln (prepared by adding 20 ml of 1 *N* sodium hydroxide to 1 l of the borate buffer described under **b)** to the washed petroleum ether solution of the reagent and shake. Remove the buffer soln and repeat about 3–4 times or until most of the color has left the petroleum ether layer. Store in refrigerator.

(d) *Dithizone soln.*—Dissolve 0.5 g of dithizone in 600–700 ml of carbon tetrachloride (redistilled is unnecessary), filter into a separatory funnel containing 2.5–3.0 l of 0.02 *N* ammonium hydroxide, shake well, and discard the carbon tetrachloride layer. Shake with 50-ml portions of redistilled carbon tetrachloride until the carbon tetrachloride phase as it separates has a pure green color. Add 1 l of carbon tetrachloride and acidify slightly with hydrochloric acid. Shake the dithizone into the carbon tetrachloride and separate. Store in a cool, dark place.

(e) *Hydroxylamine-acetate buffer.*—Dissolve 10 g of hydroxylamine hydrochlo-

ride and 9.5 g of anhydrous sodium acetate in 500 ml of redistilled water. The *p*H should be between 5.0 and 5.2.

(f) *Ammonium citrate soln, 40%.*—Dissolve 800 g of lead-free citric acid in 600 ml of distilled water, then add slowly, with stirring 900 ml of concentrated ammonium hydroxide. Adjust the *p*H to 8.5 if necessary. Dilute to 2 l and extract with 10-ml portions of dithizone soln in carbon tetrachloride until the aqueous phase stays orange colored and the carbon tetrachloride phase remains predominantly green. Then extract the soln with carbon tetrachloride until all the orange color is removed.

(g) *Ligroin.*—Ligroin obtained from Eastman Kodak Company (b.p. 70° to 90°C.) was used. Distill over dilute, alkaline potassium permanganate, then wash by shaking vigorously 3 times with distilled water in a separatory funnel.

(h) *Standard cobalt soln.*—Prepare a stock soln containing 100 mmg per ml of cobalt by heating 5 g of cobalt sulfate heptahydrate in an oven at 250°–300°C. to constant weight (6–8 hrs are sufficient). Weigh exactly 0.263 g of the cobalt sulfate and dissolve in 50 ml of redistilled water and 1 ml of concentrated sulfuric acid. Make to volume of 1 l. Transfer 5 ml of the stock cobalt soln to a 1-l volumetric flask and dilute to volume with redistilled water for the standard soln. This soln contains 0.5 mmg of cobalt per ml.

(i) *Ammonium hydroxide soln.*—Distill concentrated ammonium hydroxide into an equal volume of redistilled water.

(j) *Phenolphthalein,* 1% soln in 95% ethanol.

(k) *Perchloric acid,* reagent grade, 60%.

(l) *Carbon tetrachloride.*—Distill over calcium oxide and filter thru acid-washed filter paper.

(m) *Hydrochloric acid, 1 to 1.*—Add an equal volume of water to concentrated hydrochloric acid and distill.

(n) *Nitric acid, 1 to 1.*—Add an equal volume of water to concentrated nitric acid and distill.

(o) *Hydrofluoric acid.*—48% analytical grade.

(p) *Cupric acetate soln.*—Dissolve 10 g of C. P. cupric acetate in 1 l of distilled water.

Make all distillations from all-Pyrex stills.

B. REAGENTS FOR COPPER

(a) *Water.*—Water redistilled from Pyrex is preferred. Ordinary distilled water is seldom free of copper.

(b) *Carbamate soln.*—Purify a 0.1% aqueous soln of sodium diethyldithiocarbamate by shaking with carbon tetrachloride to remove copper and cobalt.

(c) *Standard copper soln.*—Dissolve 0.5 g of pure metallic copper in nitric acid and evaporate to dryness on steam bath. Dissolve the $CuNO_3$ with a little water and a few drops of acetic acid, evaporate to dryness, redissolve in same manner, and make up to a liter for the stock soln. Dilute 2 ml of this soln to a liter with 0.01 *N* HCl for the standard soln. Each ml contains 1 mmg of Cu.

C. CLEANING OF GLASSWARE

Wash with soap, rinse thoroly, dip in chromate cleaning soln. Rinse in tap water followed by 3 to 4 thoro rinsings in distilled water. Separatory funnels are best cleaned by filling with aqua regia and allowing to soak overnight. Rinse very thoroly. For very precise work they should be rinsed with dithizone soln.

D. PREPARATION OF SAMPLE

Weigh 8 g of dry plant tissue into a platinum evaporating dish (capacity about

70 ml). Include at least one blank determination with each set of samples ashed. Dry-ash at 500°C. overnight. Remove and cool the sample and add 2 ml of perchloric acid and 5 ml of hydrofluoric acid. Evaporate slowly on steam bath and finally heat on sand bath until fumes of perchloric acid no longer are given off. Return the sample to the partially cooled muffle and maintain at 500°C. for 1 hour. Remove the sample, cool and dissolve in 10 ml of 1:1 hydrochloric acid and warm on the steam bath. Normally a clear soln practically free from insoluble material will be obtained. Transfer the sample with redistilled water to a 100 ml volumetric flask, make to volume and mix thoroly.

E. DITHIZONE EXTRACTION

Transfer a 40-ml aliquot of the soln (d) to a 100-ml separatory funnel. (Use only vaseline as a stop cock lubricant.) Add 5 ml of ammonium citrate soln. Adjust the *p*H to 8.5 with ammonium hydroxide using phenolphthalein as an internal indicator. Formation of a precipitate may be prevented by adding more ammonium citrate. Add 10 ml of dithizone soln in carbon tetrachloride and shake vigorously for 10 min. Draw off the carbon tetrachloride phase into a beaker. It is often necessary to repeat the extraction with an additional 5 ml of dithizone soln. The extraction is complete when the aqueous phase remains orange and the carbon tetrachloride extract remains predominantly green in color. To the aqueous phase in the separatory funnel add a small quantity (2–3 ml) of carbon tetrachloride to remove the remaining dithizone soln. Without shaking draw off and add to the combined carbon tetrachloride extracts.

Add 5 ml of carbon tetrachloride to the aqueous phase. Shake for 2 min. Draw off the carbon tetrachloride phase and add to the combined extracts. If the dithizone extraction was complete the carbon tetrachloride extract at this point will be pure green in color. Repeat the extraction if necessary.

Add 2 ml of perchloric acid to the combined carbon tetrachloride extracts and reflux in a covered beaker on a hot plate until colorless. Remove the watch glass and evaporate off all perchloric acid. If free acid remains it will interfere with subsequent steps where *p*H adjustment is important. Dissolve the residue in 5 ml of 0.01 *N* hydrochloric acid. Transfer to a 25 ml volumetric flask and dilute to volume with redistilled water.

F. PREPARATION OF COBALT STANDARD CURVE

To 60-ml separatory funnels transfer the following volumes of the standard cobalt soln: 0, 1, 2, and 3 ml. Dilute to ca 20 ml with distilled water and 4 ml of 0.01 hydrochloric acid and treat as described under G, beginning with the addition of the sodium borate buffer.

G. DETERMINATION OF COBALT

Transfer a 20-ml aliquot of the soln under E to a 60-ml separatory funnel. Add 4 ml of the sodium borate buffer. Add sodium nitrosocresol soln drop by drop until all the copper present has reacted. This point is judged by the appearance of an orange color, which is distinct from the pink color of the copper complex which may be present. Add a 1-ml excess of the reagent. Add exactly 5 ml of ligroin and shake for 10 min. Remove the aqueous phase and to the ligroin add 5 ml of 1% aqueous cupric acetate soln. Shake 1 min and again remove the aqueous phase. Wash the ligroin with distilled water and finally with 5 ml of hydroxylamine sodium acetate buffer. After shaking, remove the aqueous layer in each case. Unless large quantities of cobalt are present, the ligroin will appear colorless, since most of the light absorption by the cobalt o-nitrosocresol complex occurs in the near ultraviolet.

Transfer the ligroin soln of the cobalt complex to a 5- or 10-cm absorption tube of

3–4 ml capacity and read in a photoelectric colorimeter using Corning standard thickness filters 5860 or 5860 plus 4308, or a light band as close as possible to the point of maximum absorption at 360 mμ.

H. PREPARATION OF COPPER STANDARD CURVE

To 60-ml separatory funnels transfer the following volumes of the standard copper soln: 0, 1, 3, and 5 ml. The standard will thus contain 0, 1, 3, and 5 mmg of Cu, respectively. Treat as described under I, beginning with the addition of the ammonium citrate.

I. DETERMINATION OF COPPER

Transfer a 3-ml aliquot of the soln under E to a 60-ml separatory funnel. Add 2 ml of 40% ammonium citrate. Adjust the *p*H by adding 2 drops of phenolphthalein and 7 *N* NH_4OH to give a definite pink color. Ordinarily 3–4 drops are sufficient. Add 10 ml of a 0.1% sodium diethyldithiocarbamate (occasionally it is necessary to again adjust the *p*H at this point), and 10.0 ml of carbon tetrachloride. Shake for 10 min. Remove if possible, the droplets of water on the stem of the separatory funnel by wiping dry with clean filter paper. Separate the CCl_4 layer into test tubes. Water droplets will ordinarily adhere to the sides of the tube when the soln is transferred to the cuvette (test tube type). Read in a photoelectric colorimeter against carbon tetrachloride as the reference at a wave length of 430 mμ, or a No. 554, or No. 5113 plus No. 3389 Corning glass light filter of standard thickness should be used.

NOTES

1. *Mechanical shaker:*

A rack holding 12 separatory funnels and that can be put in a mechanical shaker has been found very convenient. The construction is similar to that of a wooden funnel rack except that in addition a padded support is placed across the top to hold the separatory funnels securely in place during the shaking operation.

2. *Storage and delivery of reagents:*

All reagents are stored in Pyrex bottles. Where practical, automatic dispensing burettes with Pyrex reservoir bottles are used to eliminate the necessity of transferring solns from the storage bottle to a burette or pipette. Likewise, automatic burettes designed to fit the standard screw cap acid bottles are used.

3. *Absorption cells:*

For cobalt a matched pair of Pyrex glass absorptions cells at least 5 cm in length is required. A satisfactory cell is made by the American Instrument Company, Silver Spring, Md. Style D (horizontal) with neck for cork or rubber stopper is recommended. The outside diameter is 13 mm and the length is 50 mm. The capacity is about 3 ml. The catalog number is 5-997.

4. *Colorimeter:*

The Coleman Model 11 spectrophotometer can be used for the cobalt determination. Any instrument that would permit the use of horizontal absorption cells would be satisfactory. For the Coleman the wave length of 345 mμ is used. The null-point method is most satisfactory at this wave-length. With this instrument, the calibration curve deviates slightly from linearity, probably because of impurity of the light band. The region of the curve between 0 and 1 microgram of cobalt approaches very close to a straight line. Any standard colorimeter is suitable for copper.

Blank determination:

The total elimination of cobalt and copper contamination during the determination is extremely difficult to accomplish. Consequently blanks must be run in

exactly the same manner as is the sample. The value for the sample should be corrected accordingly.

ALTERNATE METHODS FOR COPPER AND COBALT

The following methods are suggested as alternate methods when platinum or the proper colorimeters are not available. Neither method is as precise as the preferred methods but they have given good results in the hands of capable analysts.

J. PREPARATION OF REAGENTS FOR COBALT

(*a*) *Hydrochloric acid:*

1. Approximately 0.5 *N* soln.—Dilute 40 ml of concentrated HCl to 1 liter with distilled water.

2. Approximately 6 *N* soln.—Dilute 484 ml of concentrated HCl to 1 liter with distilled water.

(*b*) *Nitric acid soln.* (1+1).—Dilute concentrated HNO_3 with an equal volume of distilled water.

(*c*) *Sulfuric acid soln.* (1+1).—Dilute concentrated H_2SO_4 with an equal volume of distilled water.

(*d*) *Nitroso-R-salt. 0.1% aqueous soln.*—Dissolve 1 g of powdered nitroso-R-salt (obtainable from Eastman Kodak Co.) in distilled water, and dilute to 1 liter.

(*e*) *Phenolphthalein. 1% ethanolic soln.*—Dissolve 1 g of phenolphthalein in 95% ethanol and dilute to 100 ml.

(*f*) *Sodium acetate.*—(C. P. or reagent quality crystals.)

(*g*) *Sodium hydroxide. 20%* soln.—Dissolve 200 g of NaOH in distilled water and dilute to 1 liter.

(*h*) *Standard cobalt solns:*

1. *Soln A.*—Dissolve .4772 g of $CoSO_4 \cdot 7H_2O$ (use Baker's analyzed reagent quality without drying) in distilled water and dilute to 1 l. This soln contains 100 mmg. of Co per ml.

2. *Soln B.*—Dilute 2 ml of soln A to 200 ml with distilled water. This soln contains 1 mmg per ml.

K. DETERMINATION OF COBALT

Weigh a 10-g sample into a porcelain dish. Add 3 ml of the H_2SO_4, dry in an oven or on a steam bath, and ignite in a muffle furnace overnight at 500°C. (If the ash remains black or dark gray, it is necessary to cool the crucible, moisten the residue with a few drops of concentrated HNO_3, and reignite until the ash is white or nearly so.) Treat the ash with 2 ml of 6 *N* HCl, add a few ml of hot water, and transfer soln and insoluble residue into a 100 ml volumetric flask. Make to volume and transfer a 90 ml aliquot of the soln to a beaker. Add 5 drops of concentrated HNO_3 and 3–4 glass beads. Evaporate to 15–20 ml on a hot plate or steam bath, taking care to avoid spattering. Cool. Add 2 ml of the nitroso-R-salt reagent with a pipet and ca 2 g of sodium acetate measured in a scoop. Heat to ca 70°C. Add 2 drops of phenolphthalein soln, and then the NaOH soln dropwise until a pink color first appears. Immediately add the 0.5 *N* HCl until the pink color is just destroyed. Heat to boiling, add 5 ml of the 1:1 HNO_3, and boil for exactly 2 min. Cool in the dark. Avoid bright light at all stages after addition of the nitroso-R-salt. Filter into a 50-ml volumetric flask, wash the filter paper twice with small portions of distilled water, make the volume, and mix. Determine the per cent of light transmitted by each soln with the photoelectric colorimeter, using the blank soln as the standard of comparison, and evaluate the cobalt present from a curve relating light transmission and concentration. This curve is prepared by carrying known quantities of Co and a blank soln thru the procedure as directed for the unknowns; determining the per cent of light

transmitted by each soln, using the blank soln as the standard of comparison; and plotting the values thus obtained against the respective concentrations.

L. PREPARATION OF REAGENTS FOR COPPER

(*a*) *Ammonium hydroxide soln.* (*1+1*).—Dilute concentrated NH_4OH with an equal volume of distilled water.

(*b*) *Carbamate soln.*—Dissolve 1 gram of sodium diethyldithiocarbamate in distilled water and dilute to 1 liter. Filter if necessary.

(*c*) *Carbon tetrachloride.*—C.P. grade.

(*d*) *Citric acid, 15% soln.*—Dissolve 150 g of C.P. citric acid in distilled water and dilute to 1 l.

(*e*) *Hydrochloric acid.*—Concentrated C.P.

(*f*) *Litmus paper.*—Neutral.

(*g*) *Standard copper soln:*

a. Soln 1.—Dissolve 0.7587 g of anhydrous $CuSO_4$ (Merck's reagent quality 99.668% pure) in distilled water and dilute to 1 l.

b. Soln 2.—Dilute 100 ml of Soln 1 to 1 l. (1 ml contains .030 mg of copper.)

M. DETERMINATION OF COPPER

Pipet an aliquot of the soln from K (usually 2–3 ml) containing 0.5–5.0 mmg of Cu into a 125 ml separatory funnel. Add 5 ml of the citric acid soln. Drop a small piece of litmus paper into the funnel, and neutralize the acid with the NH_4OH. Add 10 ml of the carbamate reagent, and exactly 5 ml of CCl_4. Shake the mixture vigorously until the aqueous layer appears colorless after the CCl_4 separates. Let the funnel stand for 20 min, dry the stem with a cotton swab, and draw off the CCl_4 layer into a clean, dry test tube. Pour the soln into another tube to remove traces of water, and stopper it to prevent excessive volatilization. Determine the per cent of light transmitted by each soln with the photoelectric colorimeter, using CCl_4 as the reference, and evaluate the copper from a curve relating light transmission and concentration. This curve is prepared by carrying known quantities of Cu and a blank soln thru the procedure in exactly the same manner as described for the unknowns; determining the per cent of light transmitted by each soln, using CCl_4 as the reference; and plotting the values thus obtained against the respective concentrations.

MATERIALS SUBMITTED FOR STUDY

Two samples were prepared for this study by grinding to about 10 mesh in a Wiley mill large quantities of alfalfa and of timothy. Experience indicated that the alfalfa would be much higher in cobalt and possibly copper than would the timothy. The oven-dry samples were mixed by rolling and were stored in several two-quart bottles. After again mixing the contents of one of these bottles, quantities of the sample were transferred to smaller bottles for shipment to the collaborators.

RESULTS OF THE COLLABORATIVE STUDY

The results reported by the collaborators are presented in Tables 1 and 2. In general, they are not very satisfactory but they represent some progress that should be useful in planning future work.

The cobalt data show a wide range of values especially in the case of the sample No. 1 (alfalfa). A number of analyses in this laboratory in-

TABLE 1.—*Cobalt content of alfalfa (sample No. 1) and timothy (sample No. 2) as reported by collaborators*

COLLABORATOR	NO. OF DETERMINATIONS	SAMPLE NO. 1		NO. OF DETERMINATIONS	SAMPLE NO. 2		REMARKS
		AV.	RANGE		AV.	RANGE	
		p.p.m.	*p.p.m.*		*p.p.m.*	*p.p.m.*	
1	3	0.12	0.11, 0.12, 0.13	3	0.03	0.02, 0.03, 0.05	Nitroso-R-salt method
2	—	0.19			0.10		
4	20	0.20	0.12–0.29	21	0.05	0.02–0.09	All ashed separately
5	2	0.13	0.14, 0.13	2	0.05	0.04, 0.06	One sample ashed
6	9	0.19	0.15–0.29				All ashed separately

TABLE 2.—*Copper content of alfalfa (sample No. 1) and timothy (sample No. 2) as reported by collaborators*

COLLABORATOR	NO. OF DETERMINATIONS	SAMPLE NO. 1		NO. OF DETERMINATIONS	SAMPLE NO. 2		REMARKS
		AV.	RANGE		AV.	RANGE	
		p.p.m.	*p.p.m.*		*p.p.m.*	*p.p.m.*	
1	3	10.0	10.2, 10.4, 9.4	3	5.0	4.6, 5.0, 5.3	
2		38.9			58.5		
3		9.6			14.4		Alternate method used.
4	6	10.2	9.3–12.9				All ashed separately.
7	10	10.1	9.2–10.3	15	5.7	4.4–7.8	All ashed separately.

dicate that this may be due to stratification in the sample. The leafy portion of the sample grinds and crushes to a very fine powder while the stems remain as relatively large pieces. The leaf contains a greater concentration of cobalt than does the stem. Consequently, a separation of the sample gives rise to erroneous results.

Fine grinding to prevent this separation results in a contamination of the sample,[5] but it is obvious that hereafter this must be resorted to in order to secure a reliable sample for collaborative study. This has not been a problem in this laboratory since relatively small quantities of material are used in the preparation of samples for analysis. Furthermore, the leaves and stems of legumes are analyzed separately in experimental work and are crushed or cut by hand to prevent contamination.

The results on sample No. 2 (timothy) indicate reasonably good agreement especially between the nitroso-R-salt method used by collaborator No. 1 and the nitrosocresol method used by the other analysts. Furthermore, both sets of data classify the timothy as being low in cobalt as compared to the alfalfa. The range in results on sample No. 2 is in general somewhat greater than desirable, but it is less than half that for sample No. 1.

The results on copper, likewise, do not represent the potentialities of the method. The agreement is good among analysts 1, 4, and 7. The results reported by No. 2 and those reported by No. 3 for sample No. 2 are high. The range of values obtained by the analysts in this laboratory does not indicate any adverse influence due to sample separation.

RECOMMENDATIONS*

It is recommended—

(1) That the Associate Referee determine the reasons for sample variation with respect to cobalt determination and correct that difficulty.

(2) That comparative analyses using both the nitrosocresol method and the nitroso-R-salt method be studied collaboratively with the objective of selecting the method most suited to routine work.

(3) That collaborative work on the carbamate method for copper be continued and extended.

[5] *Ind. Eng. Chem. Anal. Ed.*, **15**, 527 (1943).

* For report of Subcommittee A and action of the Association, see *This Journal*, **33**, 41 (1950).

REPORT ON CAROTENE AND ZINC IN PLANT TISSUE

By ERWIN J. BENNE, *Associate Referee*, and BETTY R. JOHNSTON
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CAROTENE

Very little involving new principles for determining carotene in plant tissue has been published since the literature on this subject was reviewed in the report of the Associate Referee for 1946 (1). A few articles describing modified technics for the determination of carotene in certain kinds of plant material have appeared in the literature; however, all were adaptations based on previously published principles.

Wilkes (2) reported the use of heat-treated Kieselguhr, especially Johns-Manville Hyflo Super-cel, as a chromatographic adsorbent for the routine determination of total carotene in plant materials. He stated that under proper conditions almost all non-carotene pigments were separated from a petroleum ether solution while carotenes passed through the column practically unadsorbed. Furthermore, he reported that Hyflo Super-cel could be used to separate cryptoxanthin from carotene and that it might be applicable to the separation of vitamin A from carotenoids.

Zscheile and Whitmore (3) published a procedure for the determination of carotene in both fresh and dried alfalfa plants. They suggested that the fresh plants be first blanched with boiling water and then extracted in a Waring Blendor with a mixture of acetone and Skellysolve B, in the presence of a small amount of magnesium carbonate. The carotene was separated from other pigments with a chromatographic column of activated magnesia and Hyflo Super-cel and evaluated spectrophotometrically. The procedure suggested for use with dried alfalfa plants was similar but obviously omitted the blanching process.

Nelson (4) described a method for the determination of carotene in dried grass in which bone meal was used as a chromatographic adsorbent. The carotene present was evaluated colorimetrically.

Screenivason and Vaidya (5) reported a method for determining carotene in plant materials which eliminated both saponification and phasic separation. Fresh plant material was triturated with a mixture of lime and anhydrous sodium sulphate. This dehydrated the tissue and adsorbed chlorophyll and non-carotene pigments in one operation. Carotene could then be extracted quantitatively with petroleum ether and be evaluated as gross carotene or separated into its isomerides. The method was claimed to be particularly suitable for green leafy materials, feeds and fodders and to be equally applicable to fresh fruits and vegetables.

Because of the great diversity in the structure and composition of plants, it seems doubtful that a procedure for determining carotene will ever be devised that is universally applicable to all plant species. In the

authors' experience it has frequently been necessary to alter extraction and isolation technics in order to adapt them for the determination of carotene in plants species with unusual characteristics. Consequently, it probably will never be possible to include in the Book of Methods a procedure for carotene that can be applied to all plant materials without adaptation. For this reason the authors believe that those methods, together with the supplementary information, now given in the Plant Chapter will serve quite adequately as a basis for determining carotene in a diverse variety of plant materials. Both have been used in a collaborative study (6); hence, since the tentative classification is being discontinued, the Associate Referee recommends:*

(1) That the methods for carotene, **12.75** and **12.76,** be adopted, first action.

(2) That under "Supplementary Information" the paragraph numbered "4" be changed to read as follows: The $Ba(OH)_2$ may be added in a saturated aqueous soln, which is used to bring the acetone to 85% by volume. The effectiveness of this solution for removing chlorophyll is greatly increased by adding 10 g. of NaOH to each liter.

(3) That a study of the subject be continued even though the methods are classified as official.

ZINC

Although numerous methods for determining zinc in a diverse variety of materials have been published during recent years, very few have been designed specifically for evaluating the minute quantities of this element in plants. Consequently, the authors are not aware of any that are superior to the Tentative A.O.A.C. method for this purpose. Cowling (7) reported the results of a collaborative study of this method in 1941. It was studied subsequently by Shirley, *et al.* (8,9), and their findings were published in 1948 and 1949, respectively. The former report included results of an intra-laboratory collaborative study of the tentative procedure as given in *Methods of Analysis, A.O.A.C.;* and the latter described a modification of the original procedure designed to lessen the work and time required for making an analysis.

Inasmuch as the tentative classification is being discontinued and the methods will be deleted from the *Book of Methods* unless reclassified, the Associate Referee recommends:*

(1) That the method for zinc, and its divisions which are numbered **12.24–12.30,** inclusive, be adopted, first action.

(2) That the modification of the original method as published in *The Journal* (9) be included in *Methods of Analysis, A.O.A.C.* as an alternative procedure and be adopted, first action.

(3) That a study of the subject be continued even though the methods are classified as official.

* For report of Subcommittee A and action of the Association see *This Journal,* **33,** 41 (1950).

LITERATURE CITED

(1) *This Journal*, **30**, 255 (1947).
(2) *Ind. Eng. Chem., Anal. Ed.*, **18**, 702 (1946).
(3) *Anal. Chem.*, **19**, 170 (1947).
(4) *Analyst*, **72**, 200 (1947).
(5) *Anal. Chem.*, **20**, 720 (1948).
(6) *This Journal*, **24**, 526 (1941).
(7) *Ibid.*, **24**, 520 (1941).
(8) *Ibid.*, **31**, 285 (1948).
(9) *Ibid.*, **32**, 276 (1949).

REPORT ON SPECTROGRAPHIC METHODS

By W. T. MATHIS (Agricultural Experiment Station, New Haven, Conn.), *Referee*

Questionnaires were sent to 28 laboratories reported to be doing spectrographic work on agricultural or related materials. The object was to learn if the equipment available in these laboratories would lend itself to a concerted program for trial of definite techniques, in line with my recommendation in last year's report.

To date 16 replies have been received. Of these 16 laboratories, 3 have no spectrographic equipment, 2 contemplate such work but are not yet ready for operation, and 2 are working exclusively on metallurgical problems.

A tabulation of the information from the remaining 9 laboratories, plus our own, presents a very confusing picture from the standpoint of possible collaborative work. The most disturbing part of the situation, in my opinion, is the lack of uniformity in type and capacity of power sources used by these laboratories.

To cite an instance, our laboratory is unable to obtain sufficient current with our D. C. arc unit to give satisfactory results on major elements in plant material, hence we use spark excitation exclusively for quantitative work. Only three reporting laboratories have spark sources available, and these laboratories apparently do not use these sources in their present techniques. Incidentally, it would appear that only one of the nine laboratories is determining major elements in plant material on a quantitative basis, the rest being chiefly concerned with trace elements. That particular laboratory has no spark equipment, and uses D. C. arc at a much higher current level than our D. C. arc unit can deliver. Consequently, neigher of us can use the other's technique.

These points indicate that it will be very difficult to find many laboratories that are in a position to follow exacting specifications with regard to equipment. As to preliminary preparation, electrode shapes and treatment, these conditions must be suited, at least to some extent, to the ultimate conditions inherent in the equipment combination used.

It is interesting to note that the accuracy reported by the seven of these laboratories doing quantitative work falls within about the same range, regardless of variations in equipment and techniques. In other words, it would appear that, with careful work and attention to the use of proper standards, an accuracy of about 15 per cent or better might be possible under most of the conditions cited.

The criterion for judging any spectrographic technique and physical set-up is the accuracy of duplication of individual exposures on any one sample or standard, representative of the type of material being analyzed. The variation between these results will determine the number of exposures which must be averaged to obtain a satisfactory accuracy. A desirable technique is one that will eliminate erratic results and hold this variation of duplication for a series of exposures within such narrow limits that not more than two exposures will be necessary for analysis. In my experience this can be done on plant ash solutions using spark excitation.

Once such a technique is devised, the accuracy of analysis depends upon the care used in selecting standards which match the relative composition of the samples very closely. An alternative is to establish correction factors for variation in major constituents, so that a uniform analysis curve based on arbitrary standards may be used. I feel sure, however, that such correction factors would vary widely with different techniques and equipment, and would, consequently, have to be established independently.

As we all know, quantitative spectroscopy is purely a matter of comparison of an unknown amount of an element with known amounts of that element under as nearly similar conditions as possible. The most troublesome factor in maintaining this siutation is the variation of the relative percentages of major constituents in the materials under examination. One reason the spectrograph has attained such remarkable success in the metallurgical field is because standards are available to meet almost any requirement as to relative composition, so that very accurate comparisons of samples with standards may be made.

If a step in this direction could be made in our field, so that even a few accurately analyzed samples of various types of plant materials (and possibly soils) were made available to spectrographic laboratories for use as reference standards, I believe the situation would be helped considerably. Used in conjunction with normal standardization procedure, these reference standards would aid greatly in establishing the ultimate accuracy of analysis curves, and maintain a working relationship between laboratories.

The particular techniques employed are, I believe, relatively unimportant as long as the intensity ratios obtained are accurately reproducible. As stated previously, this accuracy of duplication may easily be established by running a series of exposures on single representative samples.

Materials for these reference standards should be prepared in bulk and

subdivided after careful grinding and mixing. Portions should then be analyzed for major as well as trace elements by laboratories best suited for making the particular determinations.

Spectrographic procedure sanctioned by the A.O.A.C. could conceivably require the use of these approved reference standards in establishing curve values. The A.O.A.C. method could designate specifically the standards to be used for analysis of a particular material. Pertinent and helpful information regarding satisfactory techniques in use could be included in the method, selection of technique to be optional.

RECOMMENDATIONS*

(1) That a single collaborative sub-sample of plant material be sent each participating laboratory, with the request that at least ten exposures be made. Results of the individual determinations on each element, as obtained by their usual techniques, should be requested. Thus a comparison of various techniques from the standpoint of reproducibility will be possible. This study should include as many elements as can conveniently be determined by each laboratory.

(2) That at least two or three reference standards be prepared by the means outlined.

(3) That laboratories submitting the information requested in recommendation (1) be sent three collaborative unknowns plus the reference standards, with instructions that the analysis curves be tied in with the known values of the reference samples.

The paper entitled "A modification of the Official Micro Method for the Determination of Phosphorus Content of Plant Tissue by A. J. Sterges, L. J. Hardin, and W. H. MacIntire, was published in the February number of *This Journal*, p. 114.

ANNOUNCEMENTS

Referee Assignments, Changes, Appointment

SPICES AND CONDIMENTS:

E. C. Deal, Food and Drug Administration, New Orleans, La., has been appointed as General Referee.

DISINFECTANTS:

Michael J. Pelzar, Dept. of Bacteriology, University of Maryland, College Park, Md., has been appointed Associate Referee on Media for Disinfectant Testing.

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* For report of Subcommittee A and action of the Association see *This Journal*, 33, 42 (1950).

COAL-TAR COLORS:

W. C. Bainbridge, formerly Associate Referee on Sulfonated Phenolic Intermediates, and on Mixtures of Coal-Tar Colors for Drug and Cosmetic Use, passed away on July 6, after a long illness.

COFFEE AND TEA:

E. H. Grant, Associate Referee on Chlorogenic Acid in Coffee, has resigned.

DAIRY PRODUCTS:

L. H. Burgwald, Ohio State University, Columbus, Ohio, has been appointed Associate Referee on Phosphatase Test in Dairy Products.

GUMS IN FOODS:

The name of M. J. Gnagy should be substituted for that of Sutton Redfern, as Associate Referee on Starchy Salad Dressing.

EXTRANEOUS MATERIALS IN FOODS AND DRUGS:

Dorothy Scott, Food and Drug Administration, has been appointed in place of K. L. Harris, as Associate Referee on Dairy and Egg Products.

CORRECTIONS IN MAY JOURNAL

The subject "Pyribenzamine and Benadryl" under Synthetic Drugs, is changed to read "Diphenhydramine hydrochloride (Benadryl ®) and Tripelennamine hydrochloride (Pyribenzamine ®)".

In the report on Butacaine Sulfate (Synthetic Drugs), page 207, 25th line, "55" ml should have been "5" ml.

CONTRIBUTED PAPERS

INDIVIDUAL VOLATILE ACIDS AS INDICES OF DECOMPOSITION IN TUNA

By Fred Hillig, W. I. Patterson, and Margaret MacLean
(Division of Food,* Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

The development of chemical methods for detecting spoiled raw material in canned fishery products has long been a subject of study by this Administration. Previous papers[1,2,3,4] dealt with the determination of volatile fatty acids in canned salmon, tuna, herring roe, and sardines, as an approach to the solution of this problem. The distillation procedure used reported the results in terms of "volatile acid number" (titration of 200 ml of distillate obtained from a clarified water extract of 25 g of well-mixed canned fish, multiplied by 4). No attempt was made at that time to determine the individual volatile acids, with the exception of formic acid which was determined in the volatile acid fraction by the mercuric chloride reduction method and reported as "formic acid number" (mg of formic acid in 200 ml of distillate obtained from a clarified water extract of 25 g of well-mixed canned fish, multiplied by 4).

Subsequent to this work, Clague[5] reported on a study of volatile acid formation in Maine sardines, employing the distillation procedure.[1,4] He concluded that volative fatty acids could be used as an index of the quality of the canned sardines from the standpoint of measuring deteriorative changes which had taken place in the sardines up to the time of canning.

Sigurdsson[6] critically reviewed the literature on fish spoilage. He reported data on tyrosine, amino nitrogen, peroxide and volatile acids, for the purpose of evaluating spoilage. From his data on canned herring he concluded that there was no appreciable change in the volatile acids during processing and that, of the criteria investigated, volatile acids. appeared to be the only one applicable to detecting spoilage in canned herring.

A partition chromatographic procedure for separating a mixture of the volatile fatty acids was adopted as official, first action[7] at the 1949 meeting of the Association of Official Agricultural Chemists. This procedure was applied to the acid mixture isolated by steam distilling a clarified water extract of tuna, and it was found to be satisfactory.

The present paper presents the results of a further investigation on

* W. B. White, Chief.
[1] Clark, E. P., and Hillig, Fred, *This Journal*, **21**, 684 (1938).
[2] Hillig, Fred, and Clark, E. P., *Ibid.*, **21**, 688 (1938).
[3] Hillig, Fred, *Ibid.*, **22**, 116 (1939).
[4] ———, *Ibid.*, **22**, 414 (1939).
[5] Clague, J. A., *Food Research*, **7**, 56 (1942).
[6] Sigurdsson, G. J., *Ind. Eng. Chem., Anal., Ed.*, **19**, 892 (1947).
[7] Committee C, *This Journal*, **33**, 52 (1950).

volatile acids in tuna. In this work not only was the "volatile acid number" determined (under the same conditions as those previously reported for tuna[2]) but also formic, acetic, propionic, and butyric acids. The omission of the latter two acids from a figure simply means that none was found.

PROGRESSIVE DECOMPOSITION STUDIES

Yellowfin and skipjack tuna in good condition were selected for progressive decomposition studies. The eyes were bright and the gills were deep red in color with no odor. Small portions of the flesh, removed by incisions made an inch or two from the back bone, in the front, middle, and tail portions of the fish, had no odor. The fish were placed in wire cooking baskets, stacked on a rack, and allowed to stand on the cannery floor until decomposition took place. They were examined daily for general appearance, and for the odor of small portions of the flesh removed as already described. The fish were washed daily with running water in order to remove any slime that had been formed, and also to diminish dehydration. The organoleptic classification system employed was as follows:

Class 0 = No perceptible odor in flesh.
Class 1 = An odor which while slightly "off" is only superficial in character, not deep-seated and not repugnant.
Class 2 = A barely perceptible but deep-seated odor that is somewhat repugnant and connotes decomposition.
Class 3 = An odor similar to "2" but having enough intensity to be readily noticeable; distintinctly more repugnant than "2."
Class 4 = An odor of greater intensity and decidedly more repugnant than "3."

Some of the fish selected for the study were cooked immediately, and canned according to factory practice as regards cleaning, addition of oil and salt, and processing. As each organoleptic class was reached several fish were cooked and canned in a similar way. In some cases the cooked fish were again classified by the above system. In general, the classification tended to be lower than that of the corresponding raw fish.

The data obtained on progressive decomposition studies on yellow-fin are given in Figure 1.

There was a small quantity of volatile acids even in Classes 0 and 1, and Class 2 contained slightly more. However, the quantity found in Class 3 is several fold greater than that in Class 2, with Class 4 being still higher. The trend of the data on acetic acid is the same as that for volatile acid number. No determinable quantity of formic acid was found in Class 0, while Classes 1 and 2 contained a small quantity of this acid. More than twice as much of this acid was found in Class 3 as was present in Class 2, with Class 4 containing a still greater quantity than Class 3. It is interesting to note the close grouping of the results on individual cans in Classes 0, 1, and 2. However, when decomposition has reached the

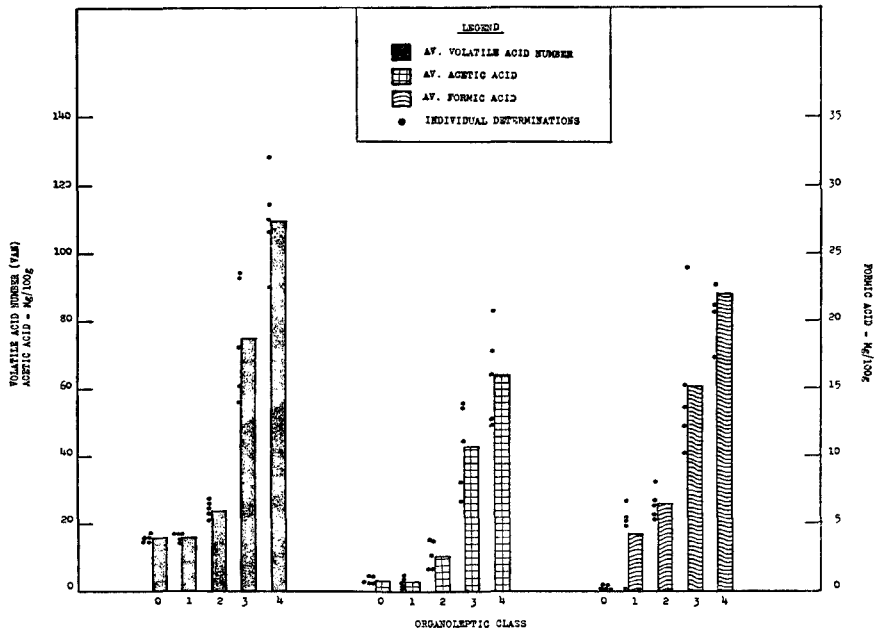

FIG. 1.—Yellowfin tuna—Progressive decomposition.

point where there can be no question about suitability for human consumption (Classes 3 and 4), the results on the individual cans are more widely separated.

The data obtained on progressive decomposition studies on skipjack are given in Figure 2.

These data are similar to those found on the progressive decomposition studies on yellowfin, except that no determinable quantities of formic acid were found in Classes 0 and 1 (none in Class 0 on yellowfin); and except that propionic acid was found in Class 4 (none in yellowfin). The larger figures obtained on Class 4, and the presence of propionic acid, indicate that the decomposition may have progressed farther than it had in Class 4, yellowfin.

FISH SPOILED ON BOAT

In Figure 3, data are presented on Classes 2 and 3, yellowfin, obtained from the butchering tables shortly after the fish were unloaded from the boat (Pack 1). The individual fish had reached the class of decomposition assigned to them between the time they were caught and the time they were delivered to the cannery.

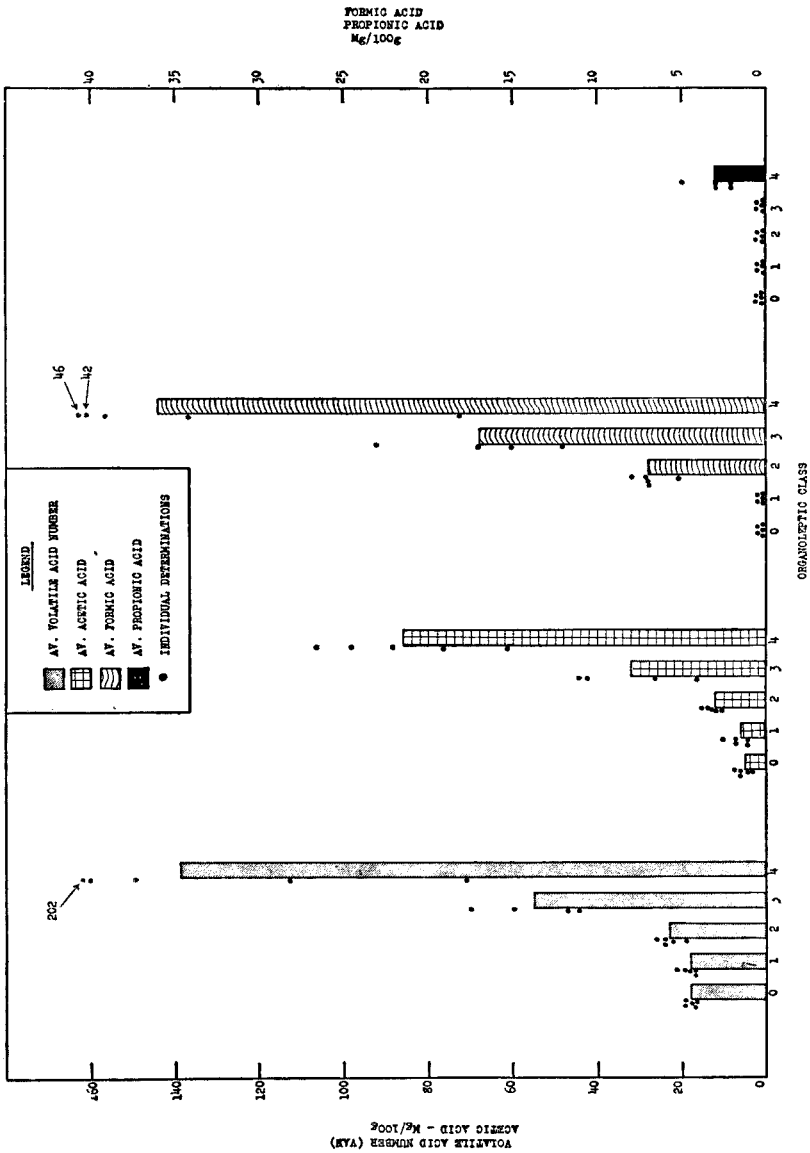

FIG. 2.—Skipjack tuna—Progressive decomposition.

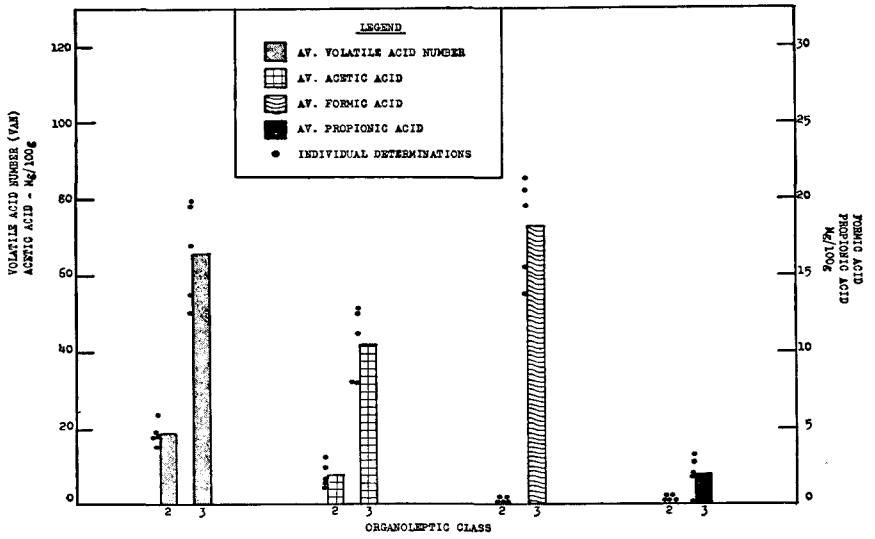

FIG. 3.—Yellowfin tuna—Boat spoilage—Pack #1.

The data for volatile acid number, acetic acid, and formic acid are very similar to those found on the progressive decomposition studies on yellowfin and skipjack. (Figs. 1 and 2.) Of particular interest is the quantity of formic acid found in the Class 3 fish (none was present in the lower classes, even in Class 2).

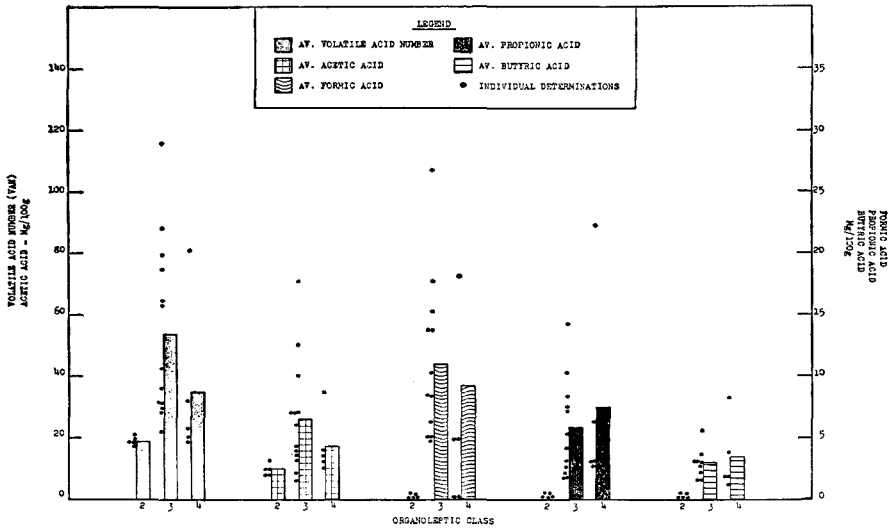

FIG. 4.—Yellowfin tuna—Boat spoilage—Pack #2.

Figure 4 presents data on Classes 2, 3, and 4, yellowfin, taken directly from the butchering tables, in a different cannery from where the fish reported in Figure 3 were obtained. (Pack 2.) The data on Classes 2 and 3 are of the same magnitude as those found in these classes on the other boat spoilage pack (Figure 3). Smaller quantities of formic and acetic acid, as well as lower "volatile acid number," were found in Class 4 than in Class 3.

In the normal packing process, the fish are gutted, placed belly side down in wire cooking baskets, and the baskets are stacked on a rack which is then wheeled into the cooking tunnel, where the fish are cooked for varying periods of time (2 to 5 hours) depending on the size. Usually

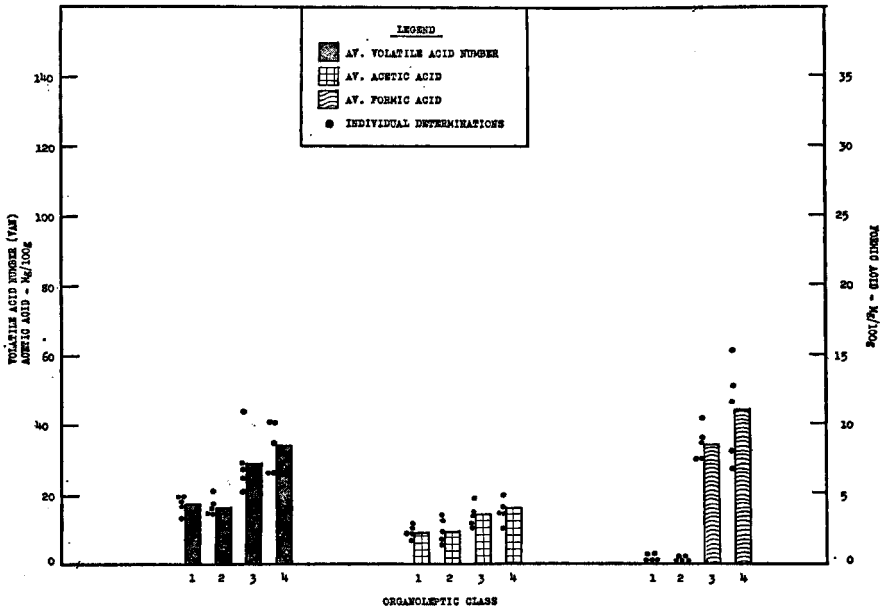

FIG. 5.—Yellowfin tuna—Progressive decomposition.

the fish reach the cooking tunnels within an hour after being gutted and washed. However, due to unforeseen conditions, such as breakdown in equipment, there is a possibility that delays of sufficient duration to result in decomposition may occur. Therefore, some progressive decomposition studies were conducted on gutted yellowfin and skipjack. No class zero fish were available; therefore the studies were started with Class 1 fish. The data obtained on such progressive decomposition studies on gutted yellowfin are given in Fig. 5.

While volatile acids were produced, the quantity was less than that found in the yellowfin allowed to decompose in the ungutted condition

(Fig. 1). Also there was no distinct spread in volatile acid number, and in acetic acid, between Classes 2 and 3. Formic acid did not appear in determinable quantities until the fish had decomposed to Class 3.

The data obtained on similar progressive decomposition studies on gutted skipjack are given in Fig. 6.

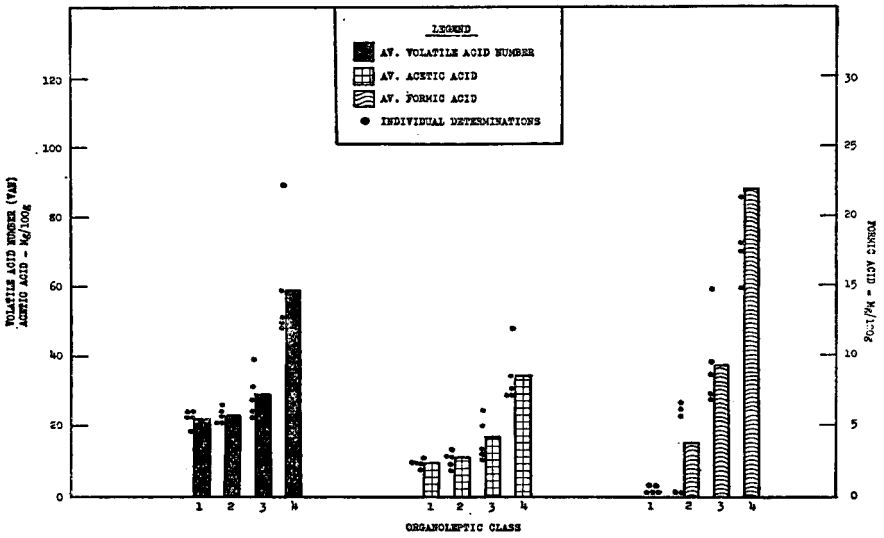

FIG. 6.—Skipjack tuna—Progressive decomposition.

Again, volatile acid number and acetic acid were lower for all four classes than in the corresponding classes of ungutted skipjack (Fig. 2). Class 4 gutted contained approximately the same quantities of both indices as Class 3 of the ungutted fish. Class 3 ungutted contained about twice as much formic acid, and Class 4 about 7 times as much as Class 2 gutted.

Table 1 summarizes the data of Figs. 1 to 4, inclusive, on volatile acids in Classes 2 and 3 ungutted tuna, representing both progressive decomposition and boat spoilage.

The averages for volatile acid number, acetic acid, and formic acid are similar for both experimental packs. No propionic or butyric acid was present in Class 3 of the progressive decomposition pack, while both these acids were present in Class 3 of the boat spoilage pack.

ACKNOWLEDGMENT

Grateful appreciation is extended to P. B. Clark, R. B. Born, L. A. Schinazi, and Eric Grey* of the Los Angeles District of the Food and

* Now with Food and Drug Service, Inc., Los Angeles, Calif.

TABLE 1.—*Volatile acids in yellowfin and skipjack tuna*

	ORGANOLEPTIC CLASS 2		ORGANOLEPTIC CLASS 3	
	PROGRESSIVE DECOMPOSITION	BOAT SPOILAGE	PROGRESSIVE DECOMPOSITION	BOAT SPOILAGE
	Volatile Acid Number (Van)			
Maximum	27	24	94	116
Minimum	19	15	44	22
Average	24 (10)	19 (10)	66 (9)	58 (18)
	Acetic Acid—mg/100 g			
Maximum	15	13	56	71
Minimum	7	4	16	8
Average	12 (10)	9 (10)	38 (9)	32 (18)
	Formic Acid—mg/100 g			
Maximum	8	Trace	24	27
Minimum	5	Trace	11	5
Average	7 (10)	Trace (10)	16 (9)	13 (18)
	Propionic Acid—mg/100 g			
Maximum	0	0	0	14
Minimum	0	0	0	0
Average	0 (10)	0 (10)	0 (9)	5 (18)
	Butyric Acid—mg/100 g			
Maximum	0	0	0	6
Minimum	0	0	0	2
Average	0 (10)	0 (10)	0 (9)	3 (18)

Figures in parenthesis are number of cans analyzed.

Drug Administration for their assistance in the preparation of the packs, to Angus Shingler of the Atlanta District, and Shirley Walden of the Baltimore District for assistance in the analysis of the samples.

SUMMARY

Canned tuna prepared from fish in good condition normally contain a small quantity of volatile acid which has been identified chromatographically as acetic acid. As decomposition progresses there is a steady increase in acetic and formic acids. When the decomposition is farther advanced, propionic and butyric acids may appear. The results indicate that individual volatile acids in canned tuna are a good index of the stage of decomposition of the raw material from which the canned product was prepared.

SUCCINIC ACID AS AN INDEX OF DECOMPOSITION IN TUNA

By FRED HILLIG, W. I. PATTERSON, and MARGARET MACLEAN (Division of Food,* Food and Drug Administration, Federal Security Agency, Washington 25, D. C.)

The desirability of more than one chemical index of decomposition in tuna or any other manufactured food requires little elaboration. Deteriorative changes in the raw material, whether caused by autolysis or microbial growth, are known to be extremely complex. Even in a single raw material, they may vary widely with the initial microbial flora, the conditions of exposure, and the like. The degradation products that contribute to the smell of decomposed raw material may not yet be determinable in their entirety, though they may be determinable in part. If the chemical index also contributes to some of the sensory evidence of decomposition, especially the smell, so much the better. In any event the index must show a reasonably good correlation with sensory criteria in the raw material, and (preferably) in the finished food as well. A single chemical index may "miss some of the bad ones" at times; it must *not* "hit some of the good ones." Obviously, the chances of missing some really decomposed material will be materially lessened if more than one chemical index is available.

In the previous paper the usefulness of various volatile acids has been shown. The present paper deals with succinic acid (non-volatile of course) as an index.

Succinic acid is known to be an intermediate product in certain natural enzyme systems,[1] and thus its possible occurrence in some decomposed foods might be anticipated. A method for its determination has already been proposed,[2] and has been applied to eggs.[3] In the method described below the succinic acid is isolated by an ether extraction of a clarified water extract of the material, separated from other acids on a partition chromatographic column, and determined by titration with standard alkali.

In the 1949 report to the Association of Official Agricultural Chemists on decomposition in fish,[4] it was stated that the work on the determination

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* W. B. White, Chief.

[1] Porter, J. R., "Bacterial Chemistry and Physiology," (1946), page 946.

[2] Claborn, H. V., and Patterson, W. I., *This Journal*, 31, 134 (1948).

[3] Lepper, Henry A., and Hillig, Fred, *Ibid.*, 31, 734 (1948).

[4] Hillig, Fred, *Ibid.*, 32, 522 (1949).

(NOTE 1). To determine the time necessary to extract all the succinic acid transfer about 20 mg succinic acid, accurately measured, to the extractor containing 20 g of solid $(NH_4)_2SO_4$, adjust the total volume in the extractor to 40 ml and proceed with the extraction as described. To determine quantity of succinic acid extracted, add 10 ml of H_2O to the extraction flask, evaporate ether on steam bath and titrate. 3 hours is usually sufficient to give recovery of 95% or better. In case it is not enough, start with another 20 mg of succinic acid and extract for a period of time that will insure recovery of 95% or better.

(NOTE 2). The following set-up can be used: a bell jar; a Gooch crucible, with removable bottom charged with a thin layer of asbestos overlaid with a small quantity of filter aid, added from a suspension in water. The asbestos was a long fibre, amphibole variety, acid and alkali washed for Gooch crucibles, and washed twice by decantation. Coarse fritted glass crucibles overlaid with a small quantity of filter aid are also satisfactory.

of succinic acid in fish indicated that this acid might be another measure of decomposition. The present paper gives some of the results obtained on tuna.

The method for the determination of succinic acid in fish and fish products follows:

DETERMINATION OF SUCCINIC ACID IN FISH PRODUCTS

APPARATUS

Continuous extractor.—*Methods of Analysis, A.O.A.C.*, 1945, sec. **22.8** (p. 304).

REAGENTS

(*a*) *Solvent.*—20% tertiary butanol in chloroform V/V.

(*b*) *Glycerol indicator soln.*—75 mg of Alphamine Red-R dissolved in 50 ml of U.S.P. glycerol. This indicator is the ammonium salt of 3,6-disulfo-beta-naphthalene-azo-N-phenyl-alpha-naphthylamine, also called R-NH_4 indicator, and is prepared according to Liddell and Rydon[5].

(*c*) *Standard barium hydroxide soln.*—0.01 *N*.

(*d*) *Phenolphthalein indicator.*—1% in alcohol.

(*e*) *Silicic acid.*

(*f*) *Ammonium hydroxide.*—ca Normal.

(*g*) *Phosphotungstic acid soln.*—20% W/V.

(*h*) *Sulfuric acid.*—ca Normal.

(*i*) *Ammonium sulfate.*

(*j*) *Sodium sulfate*, anhydrous granular.

(*k*) *Phenol red indicator.*—Rub 100 mg of phenol red in a mortar with 5.7 ml of 0.05 *N* NaOH until dissolved, then add sufficient H_2O to make the volume 100 ml.

PREPARATION OF SOLUTION

Comminute sample (include entire contents with canned products) by passing 3 times thru food chopper, mixing after each grinding. Weigh 100 g into liter Erlenmeyer flask, add about 200 ml of H_2O, shake vigorously, add 50 ml of normal H_2SO_4, and mix well. Add 75 ml of 20% phosphotungstic acid soln, make to 500 g with H_2O and shake ca 1 min. Filter thru fast flowing 24 cm folded filter paper. Transfer 250 ml of the filtrate to a 400 ml beaker and evaporate to 10 ml. If the material starts to bump when the volume becomes low, place the beaker on the steam bath and evaporate to 10 ml.

EXTRACTION OF SUCCINIC ACID AND PREPARATION OF SODIUM SUCCINATE

Place 20 g of $(NH_4)_2SO_4$ in dry extractor. Transfer the evaporated material into the inner tube of extractor thru a small funnel with sufficient H_2O to make the total volume 40 ml, and add 0.5 ml H_2SO_4 (1+1). Mix by raising and lowering the inner tube. Rinse the beaker with 50 ml of ether into the inner tube of extractor. Connect efficient condenser and proceed with the extraction as directed under **22.12**, placing 150 ml of ether in the extraction flask and extracting the material for 3 hours or for whatever period is found necessary to get complete extraction (Note 1).

To flask containing ether extract add 5 ml H_2O and expel ether on steam bath. Using a 5-ml graduated pipet, neutralize contents of flask with saturated $Ba(OH)_2$ soln (phenolphthalein indicator) and adjust the volume to 20 ml with H_2O. Add 90 ml alcohol, heat almost to boiling on steam bath, cool, add about 0.5 g of filter aid, and filter with suction thru suitable filter (Note 2). Rinse the flask with 3 portions of dilute alcohol (90 ml alcohol+20 ml H_2O) and transfer the rinsings to the cruci-

ble, sucking dry after each addition. Transfer the contents of the crucible to a 100 ml beaker with 15–20 ml H_2O, and make acid with N H_2SO_4 (congo red paper). Warm on steam bath and refilter with suction, rinsing the beaker 3 times with 10 ml portions of H_2O, transferring the rinsings to the crucible, and sucking dry between each washing. Evaporate the filtrate to ca 5 ml, neutralize with N NaOH, transfer with H_2O to a 50 ml beaker, and evaporate to dryness on steam bath.

PREPARATION OF PARTITION COLUMN

Place 5 g of silicic acid in a mortar and add 0.5 ml of freshly prepared glycerol indicator soln (more may be necessary if soln has stood for several weeks), and the maximum amount of dilute glycerol soln (1+1) that the gel will hold without becoming sticky (1–3 ml usually sufficient) and 1 drop (ca 0.05 ml) of N NH_4OH. Grind into uniform powder with pestle, make a slurry of the powder with ca 30 ml of the butanol-$CHCl_3$ solvent, and transfer to a chromatographic tube (17 mm O.D. ×250 mm or tube of ca the same dimensions) plugged at the constricted end with either cotton or glass wool, and clamped in an upright position. Apply air pressure (3–5 pounds) to the top of the tube forcing the excess solvent dropwise out of the constricted end. During the removal of the excess solvent the gel will pack down. When the suspension becomes so viscous that it will no longer pour, the column is ready for use. After the gel can no longer be poured more excess solvent can be forced from the column. As the column packs down particles of the gel will adhere to the walls of the tube until at one point the gel will leave the walls of the tube relatively clean. At this point the optimum density for the column has been reached. Do not allow the column to dry below the surface of the gel; such drying or "cracking" renders the column useless. If the column cracks before the acids have been added, the gel can be extruded from the tube, reslurried with solvent, and again poured into the tube. Add 1 ml of $CHCl_3$ containing ca 5 mg acetic acid. Apply pressure until the surface of the solvent just disappears into the gel. Add 5 ml of the solvent and apply pressure until the surface of the solvent just disappears into the gel.

ISOLATION OF SUCCINIC ACID

To the dry residue of sodium succinate in the 50-ml beaker add 3 drops of sulfuric acid (1+1) and stir with a glass rod until all particles are moistened with the acid (the material should be acid to congo red paper). Add anhydrous sodium sulfate in ½ g portions until the material is dry (not gummy). Add 2 ml of the butanol-$CHCl_3$ soln, stir and decant onto the prepared partition column, pouring it slowly down the side of the tube in order to preserve the level surface of the gel. Apply pressure until the solvent just disappears into the gel. Wash the beaker with 1 ml of the solvent and decant onto the column. Apply pressure until the solvent just disappears into the gel. Again wash the beaker with 1 ml of the solvent, pour onto the column, and with stirring rod transfer the residue in the beaker onto the column. Wash the beaker with 1 ml of the solvent and transfer onto the column. Wash the inside of the tube with 1 ml of the solvent and apply pressure until the solvent just disappears into the gel. Fill the tube with solvent and apply pressure. Allow the acetic acid band to pass out of the column. When the front of the succinic acid band reaches the constricted portion of the tube start collecting the percolate in a 50-ml graduated cylinder, and continue until the band has passed entirely from the tube, or until the lower edge of any following band reaches 2–5 mm above the narrowest portion of the constriction of the tube, and until a sufficient amount of eluate has been collected to insure removal of the succinic acid from the column (Note 3). A light placed adjacent to, but not so close as to heat the tube, increases the visibility of the

bands. Add 10 ml H_2O to the flask and titrate with 0.01 *N* $Ba(OH)_2$ (phenol red indicator) in a CO_2 free atmosphere in a flask. As the end point is approached stopper the flask and shake vigorously to completely extract the acid from the solvent phase.

1 ml 0.01 *N* $Ba(OH)_2 = 0.59$ mg succinic acid.

RECOVERY EXPERIMENTS

Table 1 gives the recoveries of known quantities of succinic acid added to 100 grams of well-mixed canned tuna. The recoveries are satisfactory.

TABLE 1.—*Recovery of succinic acid added to tuna*

SAMPLE NUMBER	ACID ADDED	ACID RECOVERED	
	mg	*mg**	*per cent*
1	2	2.5	125.0
		2.1	105.0
2	5	5.1	102.0
		5.5	110.0
3	10	8.9	89.0
		9.1	91.0
4	20	19.3	96.5
		18.8	94.0
5	40	37.0	92.5
		38.0	95.0
6	60	59.5	99.2
		58.3	97.2

* Results corrected for tuna blank.

PROGRESSIVE DECOMPOSITION STUDIES

Skipjack and yellowfin, known to be in good condition, were selected for progressive decomposition studies. Descriptions of the preparation of these packs, as well as of the organoleptic system of classification employed, is given in the preceding paper on volatile acids in fish.[5] The succinic acid data obtained on these progressive decomposition studies are given in Figure 1 of the present paper.

Similar descriptions of spoiled fish, taken directly after delivery from the boat to the cannery, appear in the same paper; and these succinic acid data also appear in Figure 1.

(NOTE 3). To insure complete removal of succinic acid from the column when there is no following band, the total amount of percolate to be collected can be established by preparing the sodium salt of succinic acid (quantity known), transferring the free acid from this salt to a column using the same reagents as in the method, and titrating a 25 ml percolate followed by successive 10 ml percolates. The total ml percolate containing succinic acid gives the amount of percolate to be collected in a given determination. The last 10 ml percolate which titrates less than 0.2 ml 0.01 *N* is to be regarded as indicating complete removal and is not to be indicated in the total.

[5] Liddell, H. F., and Rydon, H. N., *Biochem. J.*, 38, 68 (1944).

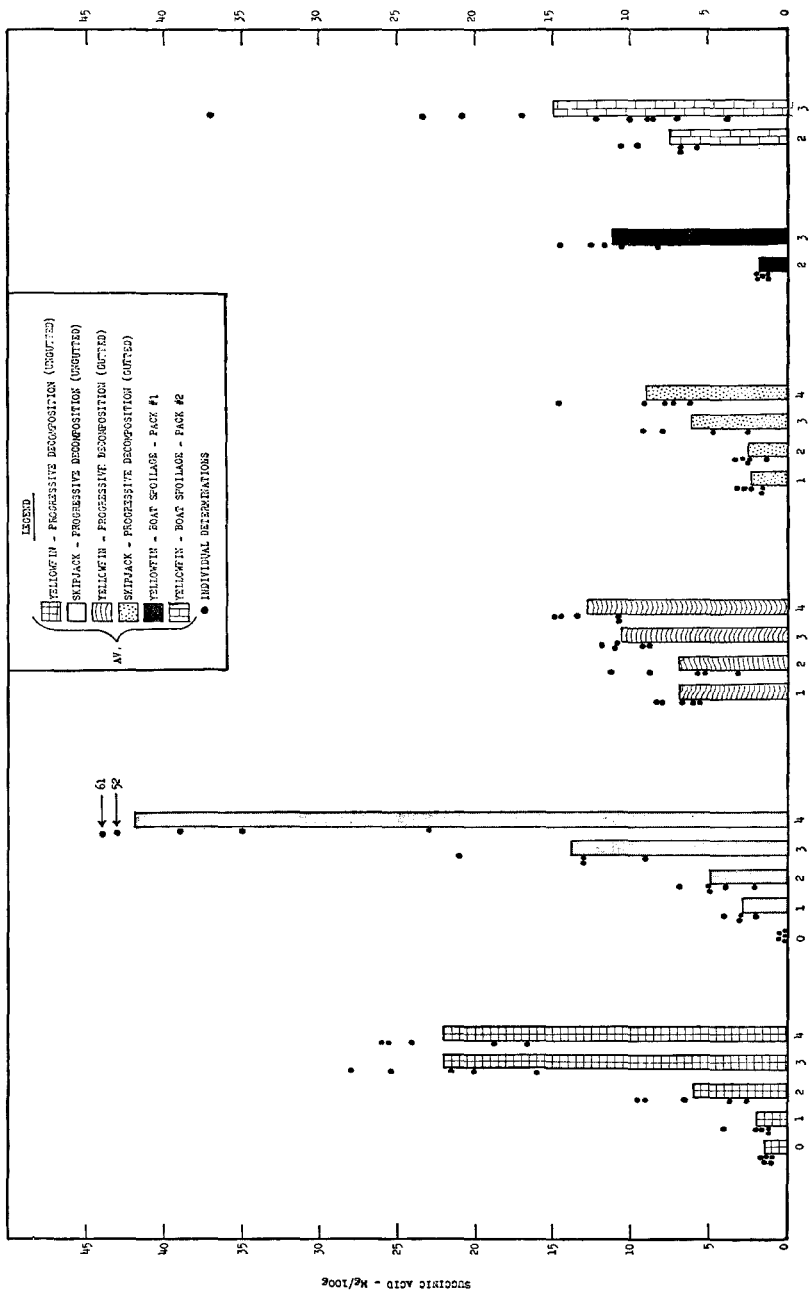

FIG. 1.—Succinic acid in ungutted yellowfin and skipjack tuna.

The figure shows that the fish, in the round, even when in good condition, contain small quantities of succinic acid. When spoilage begins, the quantity of the acid increases sharply.

Table 2 summarizes the data on succinic acid in Classes 2 and 3 (presented graphically in Figure 1).

TABLE 2.—*Succinic acid in tuna*

	ORGANOLEPTIC CLASS 2		ORGANOLEPTIC CLASS 3	
	PROGRESSIVE DECOMPOSITION	BOAT SPOILAGE	PROGRESSIVE DECOMPOSITION	BOAT SPOILAGE
	mg/100 g	*mg/100 g*	*mg/100 g*	*mg/100 g*
Maximum	10	11	28	37
Minimum	2	1	9	4
Average	6 (10)	5 (10)	19 (9)	14 (15)

(Figures in parenthesis represent number of cans analyzed.)

The average succinic acid in the fish held under progressive decomposition, and in those obtained directly from the boat, is approximately the same for each corresponding organoleptic class.

The data obtained on progressive decomposition studies on gutted yellowfin and skipjack are given in Fig. 1. (See previous paper[6] for description of this pack.)

SUMMARY

A method is proposed for the determination of succinic acid in fish and fish products. It has been applied to two normal experimental packs of fish, one of which was a progressive decomposition pack, and the other a pack in which fish of organoleptic Classes 2 and 3 were taken directly from the butchering tables immediately after delivery to the cannery. The succinic acid content of the canned tuna used in these studies correlates well with the condition of the raw material.

ACKNOWLEDGMENT

Grateful appreciation is extended to P. B. Clark, R. B. Born, L. A. Schinazi, and Eric Grey* of the Los Angeles District of the Food and Drug Administration for their assistance in the preparation of the packs, to Angus Shingler of the Atlanta District, and Shirley Walden of the Baltimore District for assistance in the analysis of the samples.

[6] Hillig, Fred, Patterson, W. I., and MacLean, Margaret, *Ibid.*, 33, 834 (1950).
* Now with Food and Drug Service, Inc., Los Angeles, Calif.

COLLABORATIVE STUDY OF A CHROMATOGRAPHIC METHOD FOR THE VOLATILE FATTY ACIDS*

By L. L. RAMSEY and S. M. HESS (Division of Food,† Food and Drug Administration, Federal Security Agency, Washington, D.C.)

Volatile fatty acids are often present in decomposed food. Those above acetic, as propionic and butyric, when present, usually represent a relatively small part of the total volatile acids. Thus, accurate measurement of these higher acids individually in such instances, by the ordinary technics of extraction and distillation, is difficult. Application of the technic of partition chromatography to the separation of mixtures of the volatile acids overcomes some of the disadvantages of other methods.

Two unknown samples in duplicate, labeled A, B, C, and D (collaborators were not told certain samples were duplicates) and consisting of an aqueous solution of the volatile acids, were submitted to 17 laboratories of the Food and Drug Administration for collaborative study; 22 analysts responded. Sample 1 (A and C) was a solution, a 2-ml aliquot of which contained 11.94 mg acetic acid, 2.91 mg propionic acid, and 3.50 mg butyric acid; sample 2 (B and D) contained in ml of solution 4.55 mg formic acid, 9.55 mg acetic acid, and 1.75 mg butyric acid. The collaborators were instructed to take a 2-ml aliquot for the determination in each case. These samples were designed primarily to simulate the mixture of acids which would be expected to occur in butter made from decomposed cream, in which a relatively small amount of butyric and/or propionic acid, with several times as much acetic acid, is characteristic. The primary purpose of this study was to test the validity of the partition chromatographic method for separation of this type of acid mixture.

The method studied is given in detail in *Methods of Analysis*, 7th Ed., 1950.

The collaborators were supplied with the following: chromatographic tubes, silicic acid, *n*-butanol, cresol red, R-NH_4 (alpha-amine Red-R) indicator, and a pure grade of formic, propionic, butyric, and valeric acids. They were directed to use a reagent grade of acetic acid from their own stock. The collaborators were also instructed to use on the 5 g. of silicic acid, in addition to the 1 ml of indicator solution specified in the method, 2.3 ml of water and 1 drop of *N* ammonium hydroxide, and to use phosphoric acid for acidification of the salts prior to chromatography. In the standardization step they were requested to report the threshold volume and the percentage recovery for each of the acids except formic; and on the collaborative samples to make a single determination and to report the threshold volume found for each acid, the identification made, and mg of

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 10–12, 1949.
† W. B. White, Chief.

TABLE 1.—*Per cent recoveries of knowns in the standardization step, about 5 mg of each acid being added*

LABORATORY	ANALYST	ACETIC	PROPIONIC	BUTYRIC	VALERIC
		per cent	*per cent*	*per cent*	*per cent*
1	a	95	103	96	94
		95	103	95	95
1	b	101	103	96	95
2	c	96	105	105	77
		84	108	98	74
		98	110	98	75
		91	101	105	80
		91	97	96	77
		94	107	106	74
3	d	94	105	96	91
		90	98	90	87
4	e	93	100	90	97
5	f	97	102	97	97
5	g	94	102	96	97
5	h	100	108	103	100
6	i	97	102	91	92
		97	103	93	93
7	j	101	99	100	93
7	k	99	101	96	90
8	l	96	103	98	94
9	m	98	104	97	98
		84	97	99	101
10	n	97	101	95	89
11	o	97	102	100	96
12	p	96	104	101	87
13	q	91	99	96	91
13	r	98	102	97	94
14	s	96	99	98	—
15	t	99	102	102	99
16	u	85	83	107	96
		—	84	93	97
17	v	88	103	94	97
Average Found		94.6	101.3	97.6	90.9
Maximum Found		101	110	106	101
Minimum Found		85	83	90	74

TABLE 2.—*Recoveries of unknowns*

LAB.	ANALYST	SAMPLE I IN DUPLICATE						SAMPLE II IN DUPLICATE					
		ACETIC		PROPIONIC		BUTYRIC		ACETIC		PROPIONIC		BUTYRIC	
		A	C	A	C	A	C	B	D	B	D	B	D
PRESENT, MG.		11.94	11.94	2.91	2.91	3.50	3.50	9.55	9.55	0	0	1.75	1.75
FOUND, MG.													
1	a	10.6	11.1	3.0	2.9	3.2	3.1	8.7	9.1	0	0	1.8	1.7
1	b	11.5	10.9	3.0	2.9	3.2	3.0	9.0	9.0	0	0	1.6	1.6
1	b	—	11.5	—	2.8	—	2.9	8.9	—	0	—	1.6	—
2	c	10.9	11.2	2.9	2.8	2.6	2.9	8.7	8.7	0	0	1.6	1.5
3	d	10.6	10.7	3.0	3.0	3.4	3.1	8.9	—	0	0	1.4	—
4	e	10.7	10.7	2.8	2.9	3.0	3.1	11.1	11.0	0	0	1.6	1.6
5	f	11.1	11.1	2.9	2.8	2.9	2.8	9.0	8.8	0	0	1.7	1.6
5	g	11.0	11.1	3.0	2.8	3.1	3.0	9.0	9.1	0	0	1.7	1.7
5	h	11.7	11.1	3.0	2.9	3.0	3.1	9.1	9.0	0	0	1.8	1.7
6	i	10.9	10.8	2.9	2.7	3.0	2.8	8.9	8.8	0	0	1.7	1.6
7	j	9.5	9.8	2.7	2.7	2.5	2.7	8.5	8.6	0	0	1.8	1.6
7	k	10.3	10.2	2.8	2.6	2.6	2.5	8.4	8.2	0	0	1.8	1.6
8	l	10.6	11.0	2.8	2.9	2.9	3.0	9.0	8.9	0	0	1.5	1.4
9	m	11.0	11.3	2.8	2.9	3.0	3.0	8.8	8.0	0	0	1.7	1.7
10	n	10.7	11.0	2.8	2.9	2.7	2.8	8.2	8.8	0	0	1.7	1.7
11	o	11.1	11.2	2.9	2.9	3.0	3.1	9.0	9.3	0	0	1.8	1.7
12	p	10.5	11.2	2.8	2.9	2.9	3.1	9.0	8.9	0	0	1.6	1.7
13	q	10.7	10.1	3.1	2.9	3.1	3.1	8.8	8.6	0	0	1.6	1.8
13	r	11.2	11.0	2.7	3.1	3.2	3.3	8.9	8.9	0	0	1.9	1.7
14	s	11.5	11.7	3.1	3.1	3.2	3.2	9.3	9.3	0	0	1.7	1.7
15	t	9.0	8.7	2.5	2.4	2.5	2.5	7.6	7.5	0	0	1.5	1.5
16	u	10.4	7.6	2.7	2.4	2.7	2.8	6.5	7.2	0	0	1.7	1.5
17	v	9.1	9.3	2.7	2.5	2.7	2.6	8.2	7.2	0	0	1.4	1.6
Avg. Found, mg.		10.64		2.84		2.93		8.74		—		1.65	
Avg. Found, %		89.1		97.5		83.7		91.5		—		94.3	
Max. Found, mg.		11.7		3.1		3.4		11.1		—		1.9	
Max. Found, %		98		107		97		116		—		109	
Min. Found, mg.		7.6		2.4		2.5		6.5		—		1.4	
Min. Found, %		64		82		71		68		—		80	

the acid found in all cases except that formic and valeric acids were not to be determined even though present.

An examination of the collaborative data indicates certain tendencies. In the standardization recovery data (Table 1) in only 6 of 95 determinations (not including valeric acid which was placed in the standardization procedure to determine whether its presence would affect the recovery of butyric acid) were the recoveries less than 90%; four acetic recoveries ranged from 84 to 88%, and two of propionic were 83 and 84%. The average recoveries for acetic, propionic, and butyric were 95, 101, and 98%, respectively. Recoveries on the unknowns (Table 2) are somewhat lower than on the knowns in the standardization step. The averages are: on duplicate samples A and C, acetic 89%, propionic 98%, and butyric 84%; and on duplicate samples B and D, acetic 92% and butyric 94%.

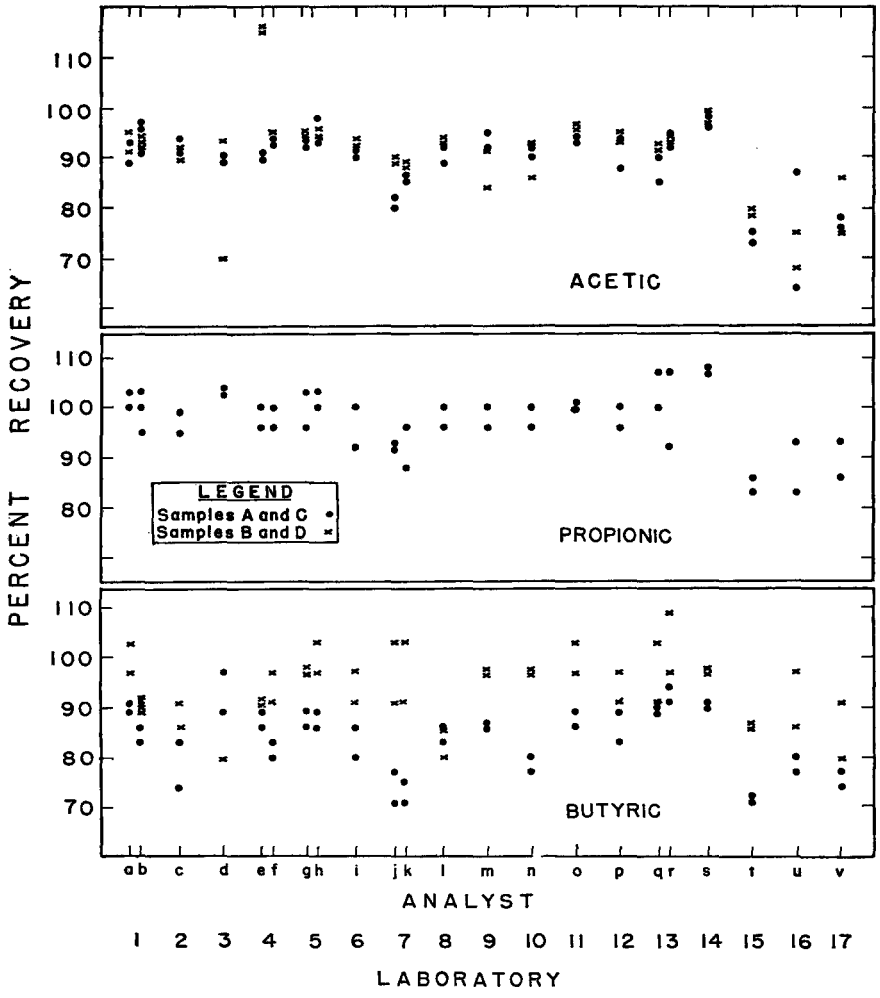

FIG. 1.—Collaborative study, volatile fatty acids.

It is to be noted that when propionic acid is absent, samples B and D, the average recovery of butyric acid is appreciably higher. Figure 1 is a graphical representation of the percentage recoveries on the unknowns. Notwithstanding the variation from maximum to minimum in no case was any acid found when it was actually absent. However, on samples B and D containing no propionic acid, 3 analysts reported a titration value for percolate collected in the region where propionic acid should elute, even though they saw no band. The directions have now been changed to

TABLE 3.—*Threshold volumes, ml.*

LABORATORY	ANALYST	ACETIC					PROPIONIC			BUTYRIC				
		SAMPLE NO.					SAMPLE NO.			SAMPLE NO.				
		STANDARDIZATION	A	C	B	D	STANDARDIZATION	A	C	STANDARDIZATION	A	C	B	D
1	a	38 40	38	41	39	36	25 24	26	29	11.3 12.3	12.7	14.0	18.0	14.0
1	b	39	39	39	37	38	25	26	28	11.2	11.3	12.4	16.8	15.5
2	c	36 36 36 36 36 39	35	37	36	36	23 23 23 24 24 22	24	26	11.5 12.0 11.5 12.5 12.5 11.5	12.5	12.5	15.0	15.0
3	d	38	36	37	37	—	24	24	25	11.5	12.0	12.0	12.5	—
4	e	40	36	34	40	43	25	24	23	12.6	11.9	12.0	13.0	16.5
5	f	32	31	33	32	34	20	21	23	10.0	11.0	11.0	14.0	14.0
	g	38	37	38	37	36	23	24	25	12.0	12.0	12.0	14.0	14.0
	h	37	35	33	35	34	24	24	22	12.0	12.0	11.0	12.0	13.5
6	i	42	39	40	37	38	24	27	30	11.4	12.0	13.0	14.0	15.0
7	j	43	44	45	45	45	28	31	31	13.4	15.0	14.7	15.0	15.0
	k	42	43	40	41	42	28	31	30	13.5	14.5	14.5	14.5	15.8
8	l	47 42 37	36	36	37	38	23 27 23	24	23	11.5 12.0 10.7	11.5	11.0	11.5	12.0

TABLE 3.—*Threshold volumes, ml—(continued)*

LABORATORY	ANALYST	ACETIC					PROPIONIC			BUTYRIC				
		SAMPLE NO.					SAMPLE NO.			SAMPLE NO.				
		STANDARDIZATION	A	C	B	D	STANDARDIZATION	A	C	STANDARDIZATION	A	C	B	D
9	m	38	37	37	36	37	24	25	25	11.3	12.0	11.8	11.9	14.4
10	n	35	39	35	32	36	23	27	20	11.0	13.0	10.0	13.0	13.0
11	o	32	30	32	31	31	20	20	21	10.0	9.5	9.5	11.0	10.0
12	p	32	31	30	31	33	20	21	19	9.5	10.0	9.5	11.0	10.5
13	q	40	—	40	—	40	26	25	28	12.0	12.0	14.0	13.0	13.5
	r	37	—	36	—	34	24	35	24	12.5	16.0	11.0	17.0	21.0
14	s	38	37	37	36	39	25	26	25	12.5	12.0	12.0	14.5	14.0
15	t	38	37	35	37	38	24	24	23	12.0	11.5	11.0	14.0	13.5
16	u	31	35	39	39	38	26	24	26	12.0	12.0	13.0	14.0	12.0
		29					20			12.0				
17	v	45	42	41	44	43	29	30	29	14.0	14.0	13.5	17.0	15.0
Average		37.7	37.0		37.3		24.0	25.4		11.8	12.2		14.0	
Maximum		43	45		45		29	35		13.5	16.0		21.0	
Minimum		30	30		31		20	19		9.5	9.5		10.0	

emphasize that an acid should be reported only when it gives a visible band on the column. In the above instances the values reported as propionic were added to the butyric acid since it is obvious that the acid collected was from the "tailing out" of the butyric acid band.

Table 3 shows the threshold volumes obtained on the knowns in the standardization step, and on the unknowns. Although the spread of values is wide, it is apparent that the threshold volume is a fairly reliable guide in making a tentative identification of an unknown acid. The reliability, of course, is directly dependent upon the analyst's ability to reproduce his own results. Several collaborators raised questions concerning the threshold volumes of the various acids. It may be well to enumerate some of the factors which affect the threshold volume: (1) the particular batch of silicic acid used, (2) weight of silicic acid used in the chromatography, (3) quantity of water added including the indicator solution, (4) quantity of acid placed on the column, (5) percentage of *n*-butanol in the chloroform, or of alcohol that may not have been washed out of the chloroform, (6) percentage of water in the silicic acid (silicic acid is somewhat hygroscopic and should be kept in a tightly covered jar); and (7) degree of packing in the chromatographic tube (use the same pressure and compress to the same point each time).

COLLABORATORS' COMMENTS

In general the collaborators appeared to have few difficulties with the method.

(1) One collaborator stated that he had trouble, first in preparing a column without its cracking; second in transferring the solvent to the column without disturbing the surface (he finally used a test tube with a lip); and third in seeing the valeric acid band.

(2) Another collaborator said that the acetic and propionic acids eluted together in his first experiments, but the difficulty cleared up later. He also stated that he obtained better results when approximately 0.01 ml, instead of 0.03 ml, of water was added to the salts prior to acidification.

(3) Another collaborator stated that the 5 g of silicic acid could not be transferred quantitatively and that some was retained on the walls where it would dry out and absorb acid. He also suggested that during hot weather there was likelihood of loss of volatile acid, a difficulty which he suggested could be overcome by placing the tubes in a beaker of cool water.

(4) One collaborator suggested a word of caution for preparing the column. If the gel is not packed evenly, if air pockets are present, or if the solvent is not introduced carefully, a jagged front may occur. This suggestion was adopted.

DISCUSSION

The data reported indicate that the proposed method is of an accuracy and precision suitable for its application to such studies as decomposition of foods.

A NEW METHOD FOR THE DETECTION OF HEATED OR RECONSTITUTED MILK*

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Consistent false positive phosphatase tests were observed in samples of ice cream products from one manufacturer of ice cream mix. It was found that, with a mix containing vanillin and unheated milk or cream, heat treatment caused the product to develop a blue color under the conditions of the phosphatase test. However, when vanillin was added to milk products which had previously been heated to 80°C., and the mixture was given the customary heat treatment, the product was then inactive to the phosphatase test. Modification of the phosphatase test by elimination of both disodium phenyl phosphate and incubation had no effect upon this color development. A procedure based on these findings was developed, and tested to determine its value as a means of detecting heated or reconstituted milk.

We have observed that the long established tests for heated milk (1, 2) based on peroxidase activity, do not detect the presence of large amounts of heated milk in pasteurized milk. Experience with the Everson test as modified by Fairbanks (3) showed that the test will detect 10% of milk reconstituted from milk powder, but is considerably less sensitive to milks reconstituted from condensed milks. A test which would detect adulteration of pasteurized milk with any reconstituted milk products would obviously be useful in law enforcement work. The proposed method follows:

METHOD

REAGENTS

Vanillin soln.—Dissolve 100 mg vanillin in 100 ml of H_2O.

Borate Buffer.—Dissolve 2.84 gm sodium borate ($Na_2B_4O_7H_2O$), and .33 gm of NaOH, in 1 liter of H_2O.

Gibbs Phenol Reagent.—Dissolve 40 mg 2,6 Dibromoquinone Chloroimide (B.Q.C.) in 10 ml 95% ethyl alcohol. Keep reagent tightly stoppered and under refrigeration.

n-Butyl Alcohol.

DETERMINATION

Transfer 0.5 ml of the sample to a 50 ml centrifuge tube. Add 1 ml of the vanillin soln. Mix. Incubate in a water bath for 5 min. at 61°C. Discontinue incubation. Add 5 ml of the borate buffer. Shake. Add 0.25 ml of the B.Q.C. reagent. Mix thoroly. Allow 15 min. for color development. Add 20 ml of *n*-butyl alcohol. Shake vigorously, then centrifuge. Determine optical density of the clear supernatant alcohol layer against a blank prepared in the same manner from a portion of the sample heated to 80°C. Readings in this laboratory were made on a Coleman Universal Spectrophotometer at 650 millimicrons.

* Presented at the Annual Meeting of the Association of Official Agricultural Chemists, held at Washington, D. C., October 10–12, 1949.

DISCUSSION OF METHOD

Concentration of the vanillin solution does not develop interfering color, but deterioration does. No blue color should develop on mixing 2 ml vanillin solution, 5 ml borate buffer, and 2 drops B.Q.C. The extracted color is stable for several days. The activity of a mixture was not changed by 30 minute heating at 100°C.

The activating agent of the test has not been identified. Negative results were obtained when milk was replaced by potato extract exhibiting high peroxidase activity, liver extract as a source of catalase, or mixtures of both. Hydrogen peroxide (1 drop of 0.1%) added before incubation greatly inhibits the color development between milk and vanillin. However, peroxide has slight effect when added with the B.Q.C. Vanillin is the only compound of several tested which was activated by milk under the conditions of the test. Several compounds related to vanillin, but without an aldehyde group, including vanillyl alcohol, produce color directly with B.Q.C. Vanillic acid has not been tried. Benzaldehyde, *p*-hydroxy benzaldehyde, and anisaldehyde produced no color when substituted for vanillin.

EXPERIMENTAL

The average optical density value of 221 samples of commercially pasteurized milk was 0.90 (Figure 1). Only five (2%) had density values below 0.55. These 5 samples were homogenized milk from 3 plants. Non-

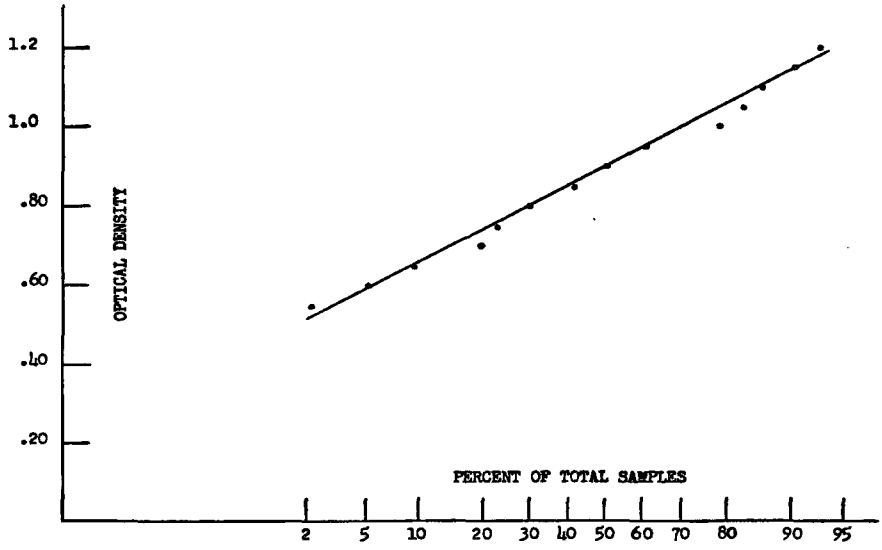

FIG. 1.—Distribution of results from 221 samples of commercially pasteurized milk.

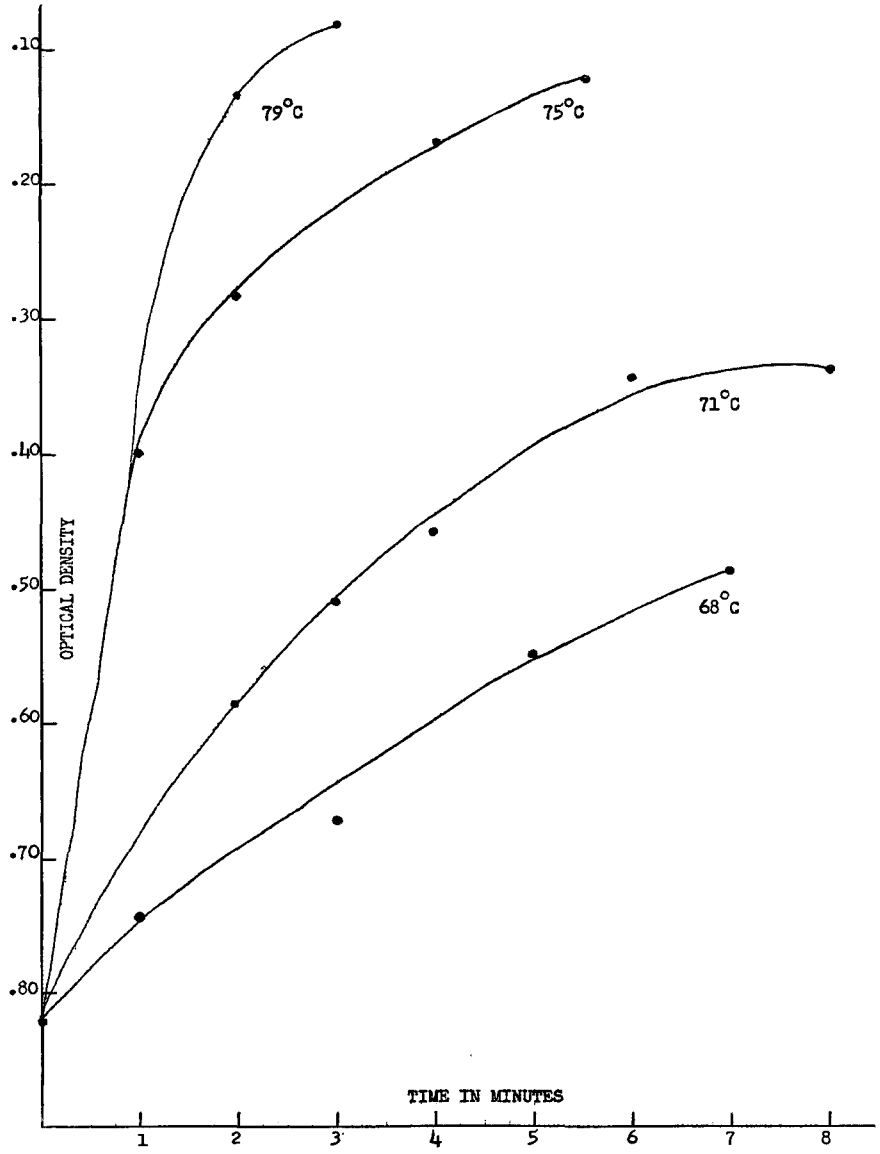

FIG. 2.—The effect of temperature on activity of pasteurized milk.

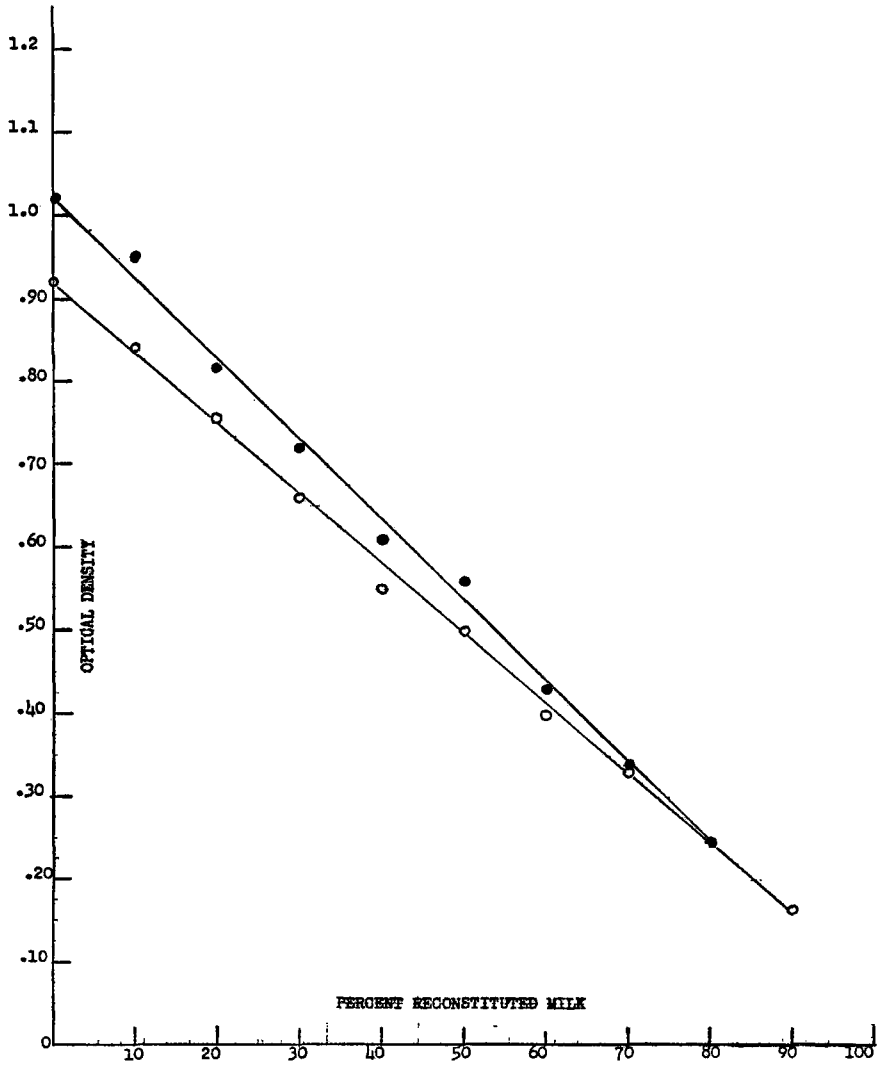

FIG. 3.—Activity of mixtures of pasteurized milk and milk reconstituted from condensed skim and inactive cream.

homogenized samples from these plants gave average values. We found that one of these plants held milk for homogenization at 150°F. for 1 hour, which probably caused the low results. Also, normally pasteurized milk from two of the plants was tested before and after homogenization and no significant difference due to homogenization was noted. These results indicate that the normal commercially pasteurized milks in our area have density values exceeding 0.55.

Pasteurized milk held at constant temperatures was tested after various holding periods (Figure 2). Inactivation was rapid at temperatures above 75°C., falling off sharply at lower temperatures. Repasteurization of 13 samples of milk reduced the average density values from 0.77 to 0.53.

Reconstituted milks prepared from 20 samples of whole- and skim-milk powder, whole and skim condensed milk, plastic cream, and butter were inactive to vanillin.

Mixtures of pasteurized whole milk and reconstituted milk were prepared in various concentrations, and density values of the mixtures determined (Figure 3). The reconstituted milk was prepared by combining condensed skim milk and plastic cream. Results using 2 whole milks of high activity are shown. Mixtures with greater than 40% adulteration had values lower than the range of the whole milk samples studied. If a sample of the whole milk used is available for comparison, 10% adulteration is detectable.

Several samples of bottled cream were tested, and activity was extremely varied. Two samples of 40% cream from "out of state" sources had no activity, probably due to extreme heat treatment, whereas 53% cream separated in the laboratory from pasteurized milk gave a density value of 1.1. It required 5 minutes heating at 75°C. to reduce the value to 0.48.

Reconstituted milks prepared from condensed skim milk and cream were tested. In Table 1, results are tabulated for 5 typical creams, and for the corresponding reconstituted milks (4% butterfat) made from each

TABLE 1.—*The effect of cream activity in reconstituted milk*

SAMPLE NO.	I WHOLE CREAM	II RECONSTITUTED MILK—CONDENSED SKIM MILK+I	III PASTEURIZED MILK	IV 50% RECONSTITUTED MILK—II+III
1	1.3	.33	.75	.56
2	1.2	.26	.70	.53
3	.77	.11	.61	.35
4	.13	.03	.64	.34
5	.05	.09	.70	.39

cream by adding condensed skim milk and water. The reconstituted milk prepared from the most active cream (No. 1) had the low density value

of 0.33. Results are likewise reported in Table 1 for 5 pasteurized milks, and for the corresponding 50% mixtures of these milks with the reconstituted skim milk and cream mixtures. The highest value (0.56) of such reconstituted milk is the minimum value found for pasteurized whole milk (Figure 1). A value greater than that minimum would have resulted if pasteurized milk of higher than average activity had been used. However, the detection of 20% addition of this more active reconstituted product would be possible with a milk of known value.

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A COMPARISON OF VITAMIN D POTENCIES COMPUTED FROM BOTH THE TIBIA AND TOE ASH OF THE SAME CHICKS

By C. I. BLISS and G. H. KENNEDY[1]

In several laboratories toe ash has given the same results as tibia ash in A.O.A.C. chick assays for vitamin D (1–5). The substitution of toes for tibiae would make it unnecessary to sacrifice the chicks at the end of the assay, or to remove and clean the bones. In the hope that an analysis of data already available would justify the substitution of toes for tibiae, the Animal Nutrition Research Council obtained from its members, data on the tibia and toe ash of the same chicks obtained in routine assays. The present paper is a report[2] of the Statistical Committee of the ANRC based upon an analysis of these data.

Data were submitted by seven laboratories, representing a total of 42 assays, in which 200 cages of chicks were fed different levels of the reference cod-liver oil, and 491 cages the test oils. The distribution of these results among the several collaborators is summarized in Table 1. The collaborating laboratories were as follows: The Borden Co., Connecticut Agricultural Experiment Station, E. I. DuPont de Nemours Co., Maryland Inspection Service, Nopco Chemical Co., White Laboratories, and Winthrop Chemical Co. All of the chicks received the A.O.A.C. diet and the assays followed the A.O.A.C. procedure. The toes were removed at the middle joint and fat extracted and dried before ashing. Each ash determination represented the bones or toes composited from the group of chicks in a single cage.

Dosage-Response Curves for the Reference Oil.—The inherent precision of an assay is no better than that of the underlying dosage-response curve,

[1] Connecticut Agricultural Experiment Station, New Haven, and E. I. DuPont de Nemours and Co., New Brunswick, N. J. The authors are greatly indebted to Miss Mary Sue Young, Department of Pharmacology, Yale University, and to Mrs. Anna R. Branchini, Connecticut Agricultural Experiment Station, for their assistance with the calculations.
[2] Presented at the annual meeting of the ANRC on October 4, 1949.

TABLE 1.—*Summary of data on tibia and toe ash of same chicks in vitamin D assays from seven laboratories*

LAB. NO.	NO. OF ASSAYS	NEGATIVE CONTROL NO. CAGES	REFERENCE OIL			TEST OILS		
			NO. DOSAGE LEVELS	NO. OF CAGES	UNITS	NO. OF OILS	NO. OF CAGES	ASSUMED UNITS
I	2	4	2–3	9	5–15	13	28	10, 15
II	5	5	3–4	25	6–18	61	115	9, 12, 15
III	2	2	4	8	6–20	2	6	6–14
IV	5	5	4–6	23	5–30	32	52	12–25
V	9	9	4	45	6.67–22.5	46	67	6.67, 10
VI	10		3–4	42	5–14.14	101	101	10
VII	10		4–5	48	8–27.4	33	122	8–27.4
Total	43			200			491	

so that the first comparison was of the dosage-response curves for the reference standard. A straight line was computed separately for each assay, relating per cent ash to the log-dose of vitamin D. Any one curve was based upon so few cages that the data from each laboratory were pooled for comparing tibiae and toes. The individual curves were first tested for homogeneity in slope. With the omission of four assays, as noted in Table 2, the remaining curves in each laboratory agreed within the sampling error, both in slope and in the standard deviation of the group ash percentages about the fitted lines. These two statistics from the individual curves of each assay were then combined to obtain the results shown in Table 2.

Both the slope (b) and the standard deviation (s) were decidedly smaller for toes than for tibiae. The error of an assay, however, is proportional to the ratio of these two terms, so that $\lambda = s/b$ was computed from the pooled data for each laboratory. In three laboratories, repre-

TABLE 2.—*Dosage-response curves for reference cod liver oil*

LAB. NO.	NO. OF CURVES RETAINED	D.F.	TIBIA ASH			TOE ASH		
			STANDARD DEVIATION s	SLOPE b	$(s/b = \lambda)$ $\lambda \pm s\lambda$	STANDARD DEVIATION s	SLOPE b	$s/b = \lambda$ $\lambda \pm s\lambda$
I	2	5	1.336	16.01	.084 ± .033	.723	7.89	.092 ± .038
II	4*	13	.759	17.88	.042 ± .009	.266	6.68	.040 ± .008
III	2	4	.969	17.21	.056 ± .021	.466	6.01	.078 ± .030
IV	4*	10	1.486	13.84	.107 ± .028	.967	8.21	.118 ± .032
V	8*	24	.718	13.85	.052 ± .008	.326	6.76	.048 ± .007
VI	10	22	1.391	16.10	.086 ± .015	.466	8.07	.058 ± .009
VII	9*	25	.944	15.59	.061 ± .009	.421	7.36	.057 ± .008

* One assay omitted to obtain homogeneity in the composite curve for the laboratory. In the omitted assays the per cent ash in the negative controls showed poor depletion and the response at all levels of vitamin D supplement approached a normal bone ash with an abnormally small slope.

senting a total of eight dosage-response curves, λ was smaller for tibiae than for toes. In the remaining four laboratories, with 31 curves, toes had the smaller λ. This suggests that, as an assay criterion, toes were as precise as tibiae.

These values of λ may be used to predict the standard error of the log-potency M for an assay in which the mean response of the unknown is equal to that on the standard. The required value was computed as

$$\text{predicted } s_M = \lambda \sqrt{\frac{1}{N_s} + \frac{1}{N_u}},$$

where N_s is the number of cages on the reference oil, or standard, and N_u is the number of cages on the test oil, or unknown. This equation has

TABLE 3.—*Comparison of assay precision for a single cage on the assay oil as predicted from λ for the dosage-response curves in Table 2 and as estimated from the observed differences in M when computed from the tibia ash and from the toe ash of the same chicks*

LAB. NO.	s_M PREDICTED FROM λ IF OBSERVED $M=0$			COMPUTED FROM $(M_{tib}-M_{toe})$	
	AVERAGE NO. CAGES ON STANDARD	TIBIA ASH	TOE ASH	NO. OF CAGES	ESTIMATED s_M
I	4.5	.092	.101	28	.049
II	5	.046	.044	114	.085
III	4	.063	.087	6	.051
IV	4.6	.118	.130	52	.100
V	5	.057	.053	67	.047
VI	4.2	.096	.064	101	.044
VII	4.8	.067	.063	122	.048

been solved for both tibia ash and toe ash (the average number of cages used on the standard in each laboratory is represented by $N_s(=4$ to $5)$; and the single cage on the unknown by $N_u(=1)$). Although the predicted standard errors varied considerably from one laboratory to another (Table 3), there was no consistent difference between tibiae and toes.

The Standard Error from the Comparison of Assayed Potencies. To compare the assay performance of tibia ash and toe ash, the log relative potency M was computed for each criterion from the data of every cage which received a test oil, or unknown, in the ration. Each M was computed by the equation

$$M = \bar{x}_s - x_u + \frac{y_u - \bar{y}_s}{b},$$

where $\bar{x}_s$ and $\bar{y}_s$ are the mean log-dose and response, respectively, on the standard; x_u is the assumed log-dose of unknown in AOAC units; y_u is the

observed response in a single cage on the unknown; and b is the slope of the dosage-response line. Some test oils were fed to more than one cage of chicks in the same assay, and ordinarily potency would be computed in such cases from the data of all cages on the same unknown. However, in order to have as many comparisons of potency as possible, each cage has been considered as a separate unit without regard to other cages in which the same test oil served as the supplement.

The problem then arose as to what slope should be used in computing each M. Because of the initial heterogeneity among assays in the slope of the standard curve in each of four laboratories, it seemed undesirable to compute the M's with the "laboratory slope." However the dosage-response curve for the standard in a single assay usually had relatively little precision. To increase the stability of b, the information on slope from test oils administered concurrently at more than one dosage level has also been included in determining an "assay slope" in computing the M's in each assay.

Two log-potencies (M) were thus available for each cage, one based upon the tibia ash and the other upon the toe ash. If the toes gave the same answer as the tibiae, the two estimates should agree. For a quantitative comparison of these separate estimates, a difference was determined for each pair as $M_{\text{tib}} - M_{\text{toe}} = D$. The variance of a single M was computed from these differences by summing their squares and dividing by twice the number of differences. Its square root was another estimate of s_M. Since each difference represented different bones of the same chicks, this second estimate should be less than that based upon the variation among different cages in the same assay.

The s_M computed from all of the differences in each laboratory is shown in the last column of Table 3, for comparison with that computed separately from the dosage-response curves for tibiae and for toes. With the exception of Laboratory II, the s_M's based upon the difference in the M's from the same chicks were less, as would be expected, although the difference was not striking. The data from Laboratory II included several aberrant results which were omitted in computing the slope of the standard and the predicted s_M, but not in determining the figure in the last column. It is evident that the potencies computed from the toe ash were in substantial agreement with those determined from the tibia ash.

Analysis of the Differences in Log-Potency.—The differences in the two estimates of the log-potency for each cage, were next examined for bias. Did the toes lead to a significantly larger or smaller estimate of potency than tibiae, either in individual assays or in different laboratories? This has been tested by computing the mean difference ($\overline{D}$) for each assay and the standard deviation about this mean. The statistic t (6) was used to test the significance of the departure of the observed $\overline{D}$ from the value of 0, since $\overline{D}$ would be zero if the two estimates agreed perfectly.

The results are shown in Table 4. In the 43 separate assays, 25 showed

TABLE 4.—*Analysis of the difference (D) in log-potency as computed from the tibia ash (M_{tib}) and from the toe ash (M_{toe}) of the same chicks*

ASSAY NO.	ASSAY SLOPE FOR COMPUTING M'S		$D=(M_{tib}-M_{toe})$				t TEST FOR D EXP. AT $S(M)=0$	t TEST FOR TREND OF D ON $M_{tib}+M_{toe}$
	TIBIAE	TOES	NO. f	MEAN $\bar{D}$	S.D. ABOUT $\bar{D}$ s_D	$\bar{D}/s_{\bar{D}}=t$		
I-1	16.97	8.24	10	−.0666	.0715	2.95[1]	2.72[1]	.98
2	15.92	8.25	18	−.0369	.0350	4.47[2]	3.49[1]	.07
T			28	−.0475	.0518	4.85[2]	5.17[2]	1.63
II-1	22.54	7.85	22	−.0175	.0604	1.36	.20	2.19[1]
2	15.83	9.76	23	−.0788	.0808	4.68[2]	3.07[1]	.54
3	20.74	5.16	25	−.0135	.1095	.62	.24	7.41[2]
4	13.06	3.95	24	.0477	.1877	1.24	.91	5.14[2]
5	17.10	5.69	20	−.0506	.0656	3.45[1]	4.21[2]	2.36[1]
T			114	−.0211	.1193	1.89	2.07[1]	5.69[2]
III-1	18.04	6.30	3	−.0540	.0652	1.43	1.50	1.75
2	19.59	8.58	3	−.0583	.0432	2.34	7.67	8.11
T			6	−.0562	.0495	2.78[1]	3.79[1]	4.25[1]
IV-1	14.35	6.63	18	.1198	.1250	4.07[2]	2.92[1]	.96
2	24.66	12.52	9	.0104	.0554	.56	.09	.93
3	12.61	10.36	6	−.0118	.0691	.42	.22	1.85
4	6.60	3.05	13	.1152	.1668	2.49[1]	2.52[1]	1.27
5	9.84	6.52	6	.0502	.0701	1.75	1.46	.06
T			52	.0765	.1209	4.56[2]	4.57[2]	.72
V-1	13.59	6.75	1	−.0108				
2	12.81	5.83	6	.0423	.0620	1.67	.43	.33
3	17.79	7.82	11	.0434	.0407	3.54[1]	3.34[1]	.11
4	13.57	5.99	12	.0242	.0398	2.11	1.84	1.16
5	12.70	6.24	4	.1518	.0563	5.39[1]	2.26	.47
6	14.51	5.91	9	.0409	.0404	3.04[1]	2.78[1]	.08
7	14.01	5.83	3	.0210	.0471	.77	.21	.22
8	13.24	6.67	11	.0185	.0418	1.47	1.52	.75
9	11.36	5.75	10	−.0340	.0611	1.76	1.71	1.69
T			67	.0285	.0602	3.87[2]	3.90[2]	3.08[1]
VI-1	14.35	8.37	11	−.0546	.0418	4.33[1]	5.26[2]	1.98
2	18.38	8.53	7	.0186	.0404	1.22	1.80	1.30
3	10.60	6.78	8	.0931	.0537	4.90[1]	3.63[1]	.21
4	16.84	7.38	11	.0195	.0419	1.54	1.32	.12
5	22.67	8.92	10	.0023	.0893	.08	.02	3.60[1]
6	12.50	6.92	11	.0081	.0587	.46	1.23	1.30
7	14.02	9.13	11	.0251	.0406	2.05	3.34[1]	2.95[1]
8	17.36	9.06	11	.0044	.0575	.25	.09	.64
9	15.92	7.03	10	−.0352	.0387	2.88[1]	2.55[1]	1.75
10	17.87	8.57	11	.0043	.0530	.27	1.63	2.01
T			101	.0061	.0623	.98	1.10	1.26

TABLE 4—(*continued*)

ASSAY NO.	ASSAY SLOPE FOR COMPUTING M's		$D=(M_{tib}-M_{toe})$				t TEST FOR D EXP. AT $S(M)=0$	t TEST FOR TREND OF D ON $M_{tib}+M_{toe}$
	TIBIAE	TOES	NO. f	NO. $\overline{D}$	S.D. ABOUT $\overline{D}$ s_D	$\overline{D}.s_{\overline{D}}=t$		
VII-1	15.13	7.56	10	−.0043	.0373	.36	.67	.94
2	15.67	6.60	9	.0426	.0535	2.39[1]	1.50	.67
3	14.35	8.04	11	.0005	.0413	.04	.79	.96
4	14.97	7.26	8	−.0325	.0595	1.54	1.47	.59
5	13.47	6.97	8	−.0710	.0332	6.05[2]	5.72[1]	.50
6	14.55	8.38	8	.0574	.0192	8.46[2]	10.75[2]	2.45[1]
7	14.85	6.98	8	−.0378	.0359	2.98[1]	3.14[1]	1.48
8	14.30	7.93	20	.0989	.0684	6.47[2]	4.43[2]	.89
9	13.97	7.13	20	.0130	.0489	1.19	.63	.10
10	14.95	8.39	20	−.0056	.0358	.70	.66	.05
T			122	.0147	.0663	2.45[1]	.82	2.19[1]

[1] Statistically significant at $P<.05$.
[2] Statistically significant at $P<.001$.

a positive mean discrepancy, and 18 a negative mean discrepancy. Eleven of these differed significantly from zero at the $P<.05$ level, and six at the $P<.001$ level, of significance. Nine of the 17 assays having a significant mean discrepancy, assayed 22% higher (weighted mean $\overline{D}=.0867$) for tibiae than for toes. In the other eight the toes showed 14% higher (weighted mean $\overline{D}=-.0553$) than the tibiae. In four laboratories representing more than 80% of the assays, the positive and negative discrepancies so balanced one another that the mean discrepancy between the two criteria was less than 7%.

The next question concerned the origin of the discrepancy. Was it related to the log-potency? This has been examined by computing separately, for each assay in each laboratory, the regression of the difference upon the sum $M_{tib}+M_{toe}$. In Laboratory II, the difference (D) increased significantly with the sum of the M's in four out of five assays, and also in the composite value for the laboratory as a whole. This trend of the difference upon the sum was significant (at $P<.05$) in only three other individual assays, and in only three other laboratory totals. In some cases the trend was positive, and in others negative. A closer examination of the data from Laboratory II showed several cases where either the standard or one of the test oils had a zero or a negative slope which contributed to the significant trend of the difference upon the sum. With this exception, and possibly also in the two small assays of Laboratory III, the difference in potency between toe and tibia assays depended very little upon whether the unknown was more or less potent than claimed.

To test whether the average difference ($\overline{D}$) in column 5 of Table 4 would disappear at $S(M)=0$, the difference expected at this level was computed

for each assay in each laboratory, in terms of the statistic t (see next to the last column of Table 4). Some of the differences decreased, and others gained, in significance. This would be expected from the usual non-significance of the trend of the difference upon the sum.

SUMMARY AND CONCLUSION

A comparison of assay results based upon the tibia ash and the toe ash of the same chicks shows that the use of either criterion leads to substantially the same results. In many assays, however, there was a small but significant difference in the response as measured by the two criteria. In comparing two independent assays in a critical region, one based upon toe ash and the other upon tibia ash, a discrepancy between them could not be attributed with certainty either to the difference in criteria or to the sampling error.

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A SIMPLE APPARATUS AS AN AID FOR DETERMINATION OF REFRACTIVE INDEX OF TRANSPARENT SOLIDS

By C. A. WOOD (U. S. Food and Drug Administration, Federal Security Agency, Philadelphia, Pa.)

There has been a recent increase in the use of the refractive index as a means for the specific identification of crystalline solids. The results obtained by the immersion method, which is generally used, are considered accurate to about $\pm .002$ (1). If not too difficult of attainment, a greater accuracy would be desirable to increase the specificity of the identification.

Gaubert (2) describes apparatus for varying the temperature of the preparation on the slide. Since the refractive index of a solid varies little with temperature, and that of liquids varies appreciably, he obtains a more accurate match of the two by warming or cooling the preparation as required. The wave length (3) of the illumination may also be varied to alter the refractive index of the liquid as an aid to a match with the solid whose variation is much less. A combination of these methods may be employed as described by Emmons (4). Accuracy of about $\pm .001$ is claimed for these methods.

This technique, however, requires rather complicated set-ups, such as special cells, monochromators, temperature controllers, and other precision apparatus besides the polarizing microscope. The simple block and

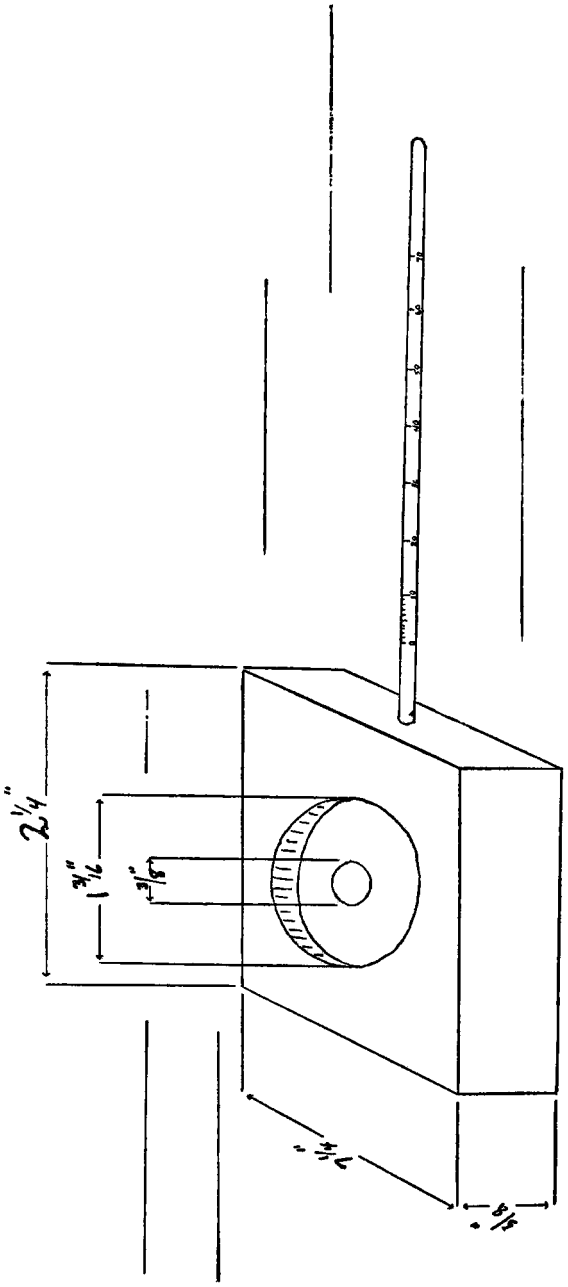

FIG. 1.—Heating block for refractive index determinations.

temperature control technique herein described yields indices within a tolerance of ±.001 and at times much closer.

The apparatus consists of a small lead block of such a size that when it heated about 15° above room temperature, it will gradually cool at a rate of 10° in 2 to 3 minutes. The Becke line on crystals is observed as the temperature of the block changes. As the refractive index of the crystal matches that of the liquid, the Becke line disappears, as does also the crystal if sufficiently free of occlusions, and then both again reappear as the temperature gradually changes.

The drawing (Fig. 1) shows the appearance of the block employed.

DETAILED DESCRIPTION OF THE BLOCK

Lead Block—$2\frac{1}{4} \times 2\frac{1}{4} \times \frac{5}{8}$ in. Diameter of upper opening, $1\frac{3}{16}$ in. extending half through the block. $1\frac{1}{16}$ in. diameter hole through the lower half.

This block is poured from melted lead into a mold previously cast and baked (100°C—6 hrs.). The composition of the mold used is, by volume, $\frac{1}{4}$ plaster of paris and $\frac{3}{4}$ asbestos. This is molded around a wooden block of the indicated dimensions.

Brass Washer—$1\frac{1}{8}$ in. diameter, $\frac{1}{16}$ in. thick, diameter of opening, $\frac{3}{8}$ in. This rests on the collar formed by the small hole through the lower half of the block.

Thermometer—Small, with slight temperature lag, of the type supplied for Abbe refractometers, generally graduated in degrees from 0–75C. This is inserted horizontally through a hole bored through the center of the block so that the bulb just touches the lower side of the brass washer.

Yellow Filter—Corning lemon yellow Shade C or equivalent.

METHOD

The crystals under test are mounted in the standard liquid whose index must be within about ±.005 (depending on the mounting liquid) of the unknown. This must be previously ascertained, if not already known, by the customary immersion technique. The mount consists of a cover glass over $\frac{3}{8}$ in. in diameter which is covered by a similar or smaller glass, with the crystals and liquid between. Drop the cover glasses on the surface of the brass ring. If the refractive index sought is below that of the mounting liquid, place the apparatus on a hot plate maintained at a low heat, and allow the block to warm to about 45°. Transfer to the microscope stage and observe the crystals at a magnification of 80 to 160 times using the yellow filter. A sharp edge should be selected, free from occlusions, properly oriented with diaphragm stopped down. Observe the Becke line as it disappears, note the temperature and as the preparation cools, again note the reappearance of the line on the same crystal edge. Take the mean of the two temperatures and calculate the refractive index by the relation.

$$I = I_1 - \left(\frac{T \times dI}{dT}\right)$$

Where I = refractive index of crystal.
I_1 = refractive index of liquid at 25°.
T = mean temperature difference −25°.

and $dI/dT = av.$ change in index of the liquid per degree of T. This value is supplied with some commercial mounting liquids (5). Table 1 gives the values for other liquids commonly used as mounting media. If this

TABLE 1.—*Refractive index—temperature coefficients*

IMMERSION LIQUID	N AT 25°C.	CHANGE IN REFRACTIVE INDEX PER DEGREE	REMARKS
Kerosene	circa 1.444	.00035	Good for organic compounds
Petroleum Oil	circa 1.475	.00040	Good for organic compounds
α monochlornaphthalene or Halowax	circa 1.624	.00045	Good for organic compounds
α monobromnaphthalene	1.656	.00048	Good for organic compounds
Methylene iodide	circa 1.735	.00070	Suitable for some organic compounds
Methylene iodide sat. with sulfur	circa 1.774	.0007	Suitable for some organic compounds
Clove Oil	circa 1.528	.00050	Suitable for some organic compounds
Cottonseed Oil	1.471	.000365	Suitable for some organic compounds
Shillaber's Liquids	1.460 to 1.640	.00043 Ave.	Good for organic compounds
Mercuric iodide in KI	1.335 to 1.720	.00033 (higher range)	Good for organic compounds
Essential Oils	—	.00045 Ave.	Dissolve many organic compounds

constant is not available it can readily be determined by checking the refractive index of the liquid involved, at approximately 20 and 30° and calculating the change per degree.

If the mounting liquid index is below that of the crystal, cool the block on a piece of ice to about 10°. Dry the bottom and proceed as above except in this case the correction to the refractive index is added.

EXPERIMENTAL RESULTS AND DISCUSSION

In order to test the accuracy of the apparatus, a number of determinations were made on inorganic salts whose refractive index is recorded to four places. The results obtained are shown in Table 2 when the block was cooled, and in Table 3 when the block was warmed. It will be noted that good agreement was obtained, the greatest deviation being −0.0007 in the case of calcite and +0.0007 with $NaClO_3$.

TABLE 2.—*Corrections obtained by cooling*

COMPOUND	IMMERSION LIQUID	REFRACTIVE INDEX CHANGE PER DEGREE C	TEMPERATURE AT WHICH BECKE LINE DISAPPEARS	TEMPERATURE WHERE LINE APPEARS	REFRACTIVE INDEX OF MOUNTING LIQUID AT 25°	CORRECTION	REFRACTIVE INDEX FOUND	REFRACTIVE INDEX IN LITERATION (6)	REMARKS
$NiSO_4 \cdot 6H_2O$	Shillaber	0.00040	22°	23°	1.5100	+.0010	1.5110	1.5109 N_ω	Crystals from H_2O
$NH_4H_2PO_4$	Monochlorbenzene	0.00045	16°	23°	1.5220	+.0025	1.5245	1.5246 N_ω	Crystals from H_2O
$NaClO_3$	Cedarwood Oil	0.00040	16°	21°	1.5122	+.0026	1.5148	1.5151 N	Crystals from H_2O
$NaNO_3$	Cedarwood Oil +Halowax	0.00044	11°	22°	1.5831	+.0039	1.5870	1.5874 N_ω	Crystals from H_2O

TABLE 3.—*Corrections obtained by warming*

COMPOUND	IMMERSION LIQUID	REFRACTIVE INDEX CHANGE PER DEGREE C	TEMPERATURE AT WHICH BECKE LINE DISAPPEARS	TEMPERATURE WHERE LINE APPEARS	REFRACTIVE INDEX OF MOUNTING LIQUID AT 25°	CORRECTION	REFRACTIVE INDEX FOUND	REFRACTIVE INDEX IN LITERATION (6)	REMARKS
KH_2PO_4	Shillaber	0.00040	30°	27°	1.5100	−.0014	1.5086	1.5090	Crystals from H_2O
$CaCO_3$	Bromonaphthalene +S	0.00048	30°	28°	1.6595	−.0019	1.6576	1.6583 N_ω	Natural calcite
$NaNO_3$	Shillaber	0.00040	34°	31°	1.5907	−.0030	1.5877	1.5874 N_ω	Crystals from H_2O
$KAl(SO_4)_2 \cdot 12H_2O$	Shillaber	0.00036	36°	34°	1.4603	−.0036	1.4567	1.4562 N	Crystals from H_2O
$NaClO_3$	Shillaber	0.00040	41°	36°	1.5200	−.0054	1.5146	1.5151 N	Crystals from H_2O

TABLE 4.—*Refractive indices obtained on organic compounds with the proposed apparatus*

COMPOUND	IMMERSION LIQUID	REFRACTIVE INDEX CHANGE PER DEGREE C	TEMPERATURE AT WHICH BECKE LINE DISAPPEARS	TEMPERATURE WHERE LINE APPEARS	REFRACTIVE INDEX AT MOUNTING LIQUID AT 25°	CORRECTION	REFRACTIVE INDEX FOUND	REFRACTIVE INDEX IN LITERATION (6)	REMARKS
Vanillin	Shillaber	.00041	13°	16°	1.5505	+.0039	1.554	1.55 N_1	Lower index-prisms
Benzoic acid	Potassium Mercuric Iodide Sol.	.0004	35°	23°	1.6130	−.0014	1.6116	1.6120 β	Crystals from H_2O, shows biaxial optic axis
Methyl parahydroxy benzoate	Bromnaphthalene and methylene iodide	.0006	11°	15°	1.6840	+.0070	1.691	1.689 β	Crystals from H_2O, shows biaxial optic axis
Saccharin	Shillaber	.00040	13°	21°	1.5300	+.0032	1.533	1.535 α	Crystals from H_2O, show inclined biaxial optic axis
Mono sodium glutamate monohydrate	Shillaber	.0041	32°	24°	1.5907	−.0012	1.590	1.593 γ	Pure commercial salt difficult to recrystallize

The method was also tested on organic compounds customarily reported with an accuracy ± 0.002 or 3 (or in the older figures somewhat greater than that). The variation from the recorded data falls within their claimed accuracy. The fact that an actual measurement is made to establish the final index figure, adds to the accuracy of the determination. Under the customary technique of mounting in a liquid of higher index and then of a lower index, the reported figure is merely an estimation based on visual inspection of the two mounts. In the case of thin crystals, as benzoic acid, such a technique may lead to unusual errors, since the visibility of the crystals is reduced by their thin habit.

CONCLUSION

A simple apparatus has been described as an aid to more simple determination of the refractive index of crystals by the immersion method. Results accurate to ± 0.001 can be readily obtained with this apparatus and technique.

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THE FRIEDRICH MICROKJELDAHL METHOD FOR NITROGEN

EFFECT OF POTASSIUM SULFATE CONCENTRATION

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The hydriodic acid procedure described by Friedrich and coworkers (1) in 1933 made possible the accurate analysis by the microkjeldahl proce-

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dure of compounds containing the various *N–N* linkages. Since the hydriodic acid method is also effective in reducing nitro and nitroso groups, it has become the most generally used procedure for the reduction of oxidized forms of nitrogen and of the *N–N* linkages that do not yield their nitrogen to the usual microkjeldahl digestion.

Friedrich's method has been modified in minor details and used successfully by Clark (2), Acree (3), Belcher and Godbert (4), Kaye and Weiner (5), and by White and Secor (6), and it has been incorporated in the Kjeldahl methods described in the standard semimicro- and microchemical textbooks (7, 8, 9, 10). The Clark modification was adopted as a tentative method by this Association in 1940. It was deleted in 1949 rather than made first action, as a result of unsatisfactory performance in the analysis of a sample of methyl orange by 12 collaborators. The method as studied differed from the Association's tentative method (11) in that the amount of potassium sulfate used was larger. (This amount was the same as that used in the first action micro method (12).)

Our results for the sample of methyl orange averaged 0.4% *N* lower by the 1949 collaborative Friedrich method than they did by this Association's tentative Friedrich method as used for eight years in this laboratory (6) and by a variation of the sealed-tube method originally described by Friedrich (1). The latter two methods gave concordant results and were later found to be in good agreement with the Dumas value (12). We also noted that results for this sample by the collaborative Friedrich method averaged 0.2% *N* lower than they did by the Association's present microkjeldahl method, official first action (12). The latter is not recommended for compounds containing any *N—N* linkage.

The poor results obtained for methyl orange with the collaborative method by ourselves and by a large majority of the others participating in the study were very disturbing, since our work showed that the compound could be analyzed accurately and simply by methods currently in use. In seeking to explain these poor results, we tabulated the kind and amount of reagents used and other critical details of representative Friedrich hydriodic acid methods. The results of this survey are presented in Table 1. This table emphasizes the similarity of the technique employed by the 1949 collaborative method and the Association's former tentative method, and it also emphasizes the very high ratio of potassium sulfate to sulfuric acid used by the former. The 1949 collaborative method employed the same high salt-to-acid ratio as does the Association's present microkjeldahl method. While this ratio is unique among commonly used micro methods, it is the same as that in the Association's official Kjeldahl-Gunning-Arnold macro method (13).

This paper deals primarily with the effect of the potassium sulfate-sulfuric acid ratio on the recovery of nitrogen from compounds that have been treated with hydriodic acid prior to the usual microkjeldahl digestion.

TABLE 1.—*Comparison of Kjeldahl nitrogen methods using hydriodic acid pretreatment*

	NIEDERL & NIEDERL (7)	PREGL-GRANT (8)	FRIEDRICH (1)	BELCHER & GODBERT (9)	A.O.A.C. (11)	1949 COLLABORATIVE METHOD (15)
Sample size (mg)	3–5	2–5	3–7	20–50	10	4–10 or 10–30
Total H_2SO_4, conc. (ml)	1	2	2	4	1.5	1 or 2
K_2SO_4 (gm)	.1	2–3 small[a] knife tips	.5 to 1[a]	1.68[a]	.5[b]	.65 or 1.3[b]
$\frac{K_2SO_4 \text{ (gm)}}{H_2SO_4 \text{ (ml)}}$	.1	[c]	.25 to .50	.42	.33	.65
Catalyst[a]	2–3 mg $Hg(Ac)_2$	Small amount $Hg(Ac)_2$	100 mg $Hg(Ac)_2$	263 mg $HgSO_4$ 53 mg Se	40 mg HgO	20 mg or 40 mg HgO
HI, d = 1.7 (ml)	1	1	1	4	1	1 or 2
Red phosphorus	2–3 mg	"few grains"	"several grains"	"pinch"	none	none
HI reflux time with sample (min.)	[c]	30	30	45	45	45
HI removed with	[c]	½ flask full H_2O +2 ml H_2SO_4 Distil off water	½ flask full H_2O +2 ml H_2SO_4 Distil off water	20 ml H_2O +2 ml H_2SO_4 Boil 45 min.	Boil off HI to 0.3 ml, add H_2SO_4 +1 ml H_2O. Distil off water and 1 or more additional 1 ml portions of water.	
Digestion time after addition of catalyst (min.)	15–20	30	until clear or 30–60	45	70–80	60

[a] Added after removal of iodine and hydriodic acid.
[b] Added prior to removal of iodine and hydriodic acid.
[c] Not specified.

METHODS

The former tentative A.O.A.C. Friedrich method (Table 1 and (11)).—Unless otherwise noted, the hydriodic acid pretreatment and the following Kjeldahl digestion were conducted according to the A.O.A.C. Friedrich method as described by Clark (2) except that (a) the samples were weighed from glass weighing tubes instead of on cigarette paper, (b) two 4-mm glass beads were used to promote even boiling, (c) evaporations with water to remove hydriodic acid and iodine were continued until the sulfuric acid condensed at least half way up in the neck of the flask, and (d) the final digestion was conducted with the ring of condensing acid about one-third the way up the neck of the flask. In certain studies the concentration of potassium sulfate and the number of distillations with water to remove the hydriodic acid and iodine were varied. Samples were digested on a gas heated rack and distilled as previously described (6, 14).

The Friedrich method (Table 1 and (1)). This method was followed only in regard to the method of removal of the iodine and hydriodic acid and time of addition of the potassium sulfate. The reagents (kind and amount), the digestion, and the distillation were the same as for the former tentative A.O.A.C. Friedrich method.

The 1949 *collaborative Friedrich method* (Table 1 and (15)).—This procedure is identical to the former A.O.A.C. tentative method for hydriodic acid pretreatment and removal and for the time of addition of the catalysts; it differs in the amounts and concentrations of reagents used and in the distillation procedure. These are similar to those employed by the Association's present microkjeldahl method (12). The final digestion time is one hour.

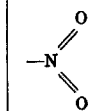
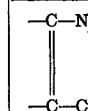
Kjeldahl method—without hydriodic acid pretreatment.—This procedure is the former A.O.A.C. microkjeldahl method (11) modified as described previously (6, 14).

DISCUSSION

Potassium sulfate is added to the Kjeldahl digest for the sole purpose of speeding up the digestion by increasing the boiling temperature of the mixture. On the macro scale 500 to 700 mg of potassium sulfate are ordinarily used per milliliter of sulfuric acid. For the micro methods, however, it has been customary to use a much lower ratio of added salt to acid. In this respect, the 650 mg of potassium sulfate per ml of sulfuric acid specified by the present A.O.A.C. micro method constitutes a marked departure from past practice. This high potassium sulfate concentration, used on the micro scale, has consistently led to nitrogen losses of the order of 0.1% *N* in our laboratory with unpretreated samples of ammonium sulfate, acetanilide, cystine, and tryptophan.

Nitrogen appears to be more easily lost during the sulfuric acid digestion if the sample has been pretreated with hydriodic acid. Therefore,

TABLE 2.—*Effect of potassium sulfate concentration on nitrogen recovery from organic compounds reduced with hydriodic acid*

COMPOUND	NITROGEN LINKAGE REQUIRING REDUCTION	HI REMOVED ACCORDING TO:	NITROGEN CONTENT, PER CENT			
			THEORETICAL	PRETREATED WITH HI		NOT PRETREATED WITH HI (6, 14)
				333 mg K_2SO_4 per ml H_2SO_4	667 mg K_2SO_4 per ml H_2SO_4	333 mg K_2SO_4 per ml H_2SO_4
2-nitroso-1-naphthol	—N=O[a]	A.O.A.C. (11)	8.09	8.07 (8.05, 8.06, 8.10)	7.95 (7.86, 7.98, 8.00)	8.09 (8.07, 8.08, 8.13)
4-nitroso-dimethylaniline	—N=O	A.O.A.C. (11)	18.66	18.70 (18.60, 18.64, 18.86)	18.18 (17.97, 18.23, 18.34)	18.32 (18.30, 18.34)
4-nitroacetanilide		A.O.A.C. (11)	15.55	15.65 (15.61, 15.68)	15.46 (15.41, 15.45, 15.53)	9.57 (9.49, 9.65)
methyl orange (impure)		A.O.A.C. (11)	[b]	11.52 (11.48, 11.50, 11.58)	11.14[c] (11.05, 11.15, 11.22)	(11.03, 11.41)
s-diphenylcarbazone, Eastman No. 4459	 and —N=N—	A.O.A.C. (11)	23.22	23.17 (23.02, 23.22, 23.27)	22.87 (22.72, 22.87, 23.01)	(11.26, 12.05)
		Friedrich (1,8)		23.22 (23.21, 23.22, 23.22)	22.78 (22.61, 22.84, 22.88)	
aminopyrine[d]		Friedrich (1,8)	18.17	18.20 (18.08, 18.24, 18.29)	18.11 (18.03, 18.11, 18.13, 18.17)	13.81 (13.78, 13.81, 13.85)

[a] nitroso⇄oxime. The tautomeric oxime does not require reduction with HI (2).
[b] 1949 A.O.A.C. collaborative sample. Nitrogen content, 11.50% (Dumas) (12).
[c] Results obtained with 1949 A.O.A.C. collaborative method (see Table 1).
[d] Sealed tube HI digestion, 3 hours at 300°C. According to Friedrich (1), compounds containing adjacent nitrogen atoms in the ring cannot be accurately analyzed by the open flask treatment, since the hydrolytic action of HI at its boiling point is not sufficient to cleave the ring.

great care should be taken not to overheat or to prolong unnecessarily the heating of samples following this treatment. Table 2 illustrates this loss for a group of samples containing nitroso, nitro, $=N-N=$, and $-N=N-$linkages which were pretreated with hydriodic acid and then digested with different concentrations of potassium sulfate. With the exception of methyl orange, all conditions of digestion and distillation were identical for the two levels of potassium sulfate used. The lower level, 333 mg of potassium sulfate per ml of sulfuric acid, is that employed by the former A.O.A.C. tentative method, and the higher level is just twice the lower and approximates the ratio used in the present A.O.A.C. micro method (12) and in the 1949 collaborative Friedrich method. Satisfactory results were obtained for each of the compounds by the former A.O.A.C. Friedrich method; and, without exception, increase in potassium sulfate concentration resulted in a significant loss of nitrogen and generally in poorer precision. These losses range from 0.09% *N* with aminopyrine (a very refractory compound) to 0.52% *N* for 4-nitrosodimethylaniline.

The data for s-diphenylcarbazone show that the hydriodic acid and iodine can be removed satisfactorily by either the Friedrich or the A.O.A.C. method.

A comparison of the apparent nitrogen content of these compounds after digestion with and without the hydriodic acid pretreatment shows that all except 2-nitroso-1-naphthol require the reduction. This material is not a typical nitroso compound, since the hydroxyl hydrogen adjacent to the nitroso group readily forms the tautomeric oxime. Oximes should not require pretreatment with hydriodic acid (2). The 2-nitroso-1-naphthol is included here to emphasize again that pretreatment and digestion by the former A.O.A.C. tentative method do not cause nitrogen loss while digestion with the higher concentration of potassium sulfate does cause a loss.

Studies with ammonium sulfate (Table 3) show that repeated boiling with water to remove hydriodic acid and iodine does not cause loss of nitrogen for either the high or low potassium sulfate concentration; however, continuing the sulfuric acid digestion for 70–80 minutes, as required for an organic compound, did cause a loss at the higher but not at the lower level. The loss noted for only one distillation to fumes at each level of potassium sulfate appears to be connected with the incomplete removal of iodine from the flask prior to the sulfuric acid digestion with the catalyst. The relatively high losses shown by hydriodic acid-pretreated and digested samples compared to those for samples receiving only the sulfuric acid digestion indicate that high potassium sulfate concentration is more likely to cause nitrogen loss from samples that have been pretreated with hydriodic acid than it is from those that have not received this treatment. Since the excessive losses occur with ammonium sulfate as well as with organic compounds, it is reasonable to suppose that they are

TABLE 3.—*Effect of potassium sulfate concentration and iodine removal on loss of nitrogen from ammonium sulfate during various digestions (loss expressed as milliliters of 0.01 N HCl)*

METHOD OF DIGESTION	DIGESTION TIME AFTER ADDING HgO	NUMBER OF DISTILLATIONS WITH WATER AND—					
		500 mg K_2SO_4 per 1.5 ml H_2SO_4			1000 mg K_2SO_4 per 1.5 ml H_2SO_4		
	min.	*1*	*2*	*3*	*1*	*2*	*3*
					Nitrogen Loss		
HI pretreatment only[a]	2	None	—	None	None	—	None
HI pretreatment and digestion with H_2SO_4 and HgO[a]	70–80	.10[c]	None	None	.27[c,d]	.13[d]	.11[d]
Digestion with sulfuric acid and HgO[b]	70–80	None	—	—	.06	—	—

[a] One mg of nitrogen added in 2 ml aliquot after 45 minute reflux and evaporation of HI to 0.3 ml.
[b] One mg of nitrogen added in 2 ml aliquot at start of digestion.
[c] Visible amounts of iodine remained in flask at time of adding HgO.
[d] Duplicates varied by more than 0.05 ml of 0.01 *N* HCl.

due to some reaction or decomposition products of hydriodic acid or of hypophosphorous acid (the preservative used in the hydriodic acid) and not to losses occurring during the conversion of the nitrogen from the organic form to ammonia. In order to determine whether the presence of hypophosphorous acid or the absence of red phosphorus (used by Friedrich but not used by the former tentative A.O.A.C. or 1949 collaborative methods—see Table 1) is responsible for the loss, s-diphenylcarbazone was analyzed by the former A.O.A.C. tentative method using all combinations of high- and low-level potassium sulfate; hydriodic acid prepared according to Clark (16) containing 0.7% v/v hypophosphorous acid and freshly distilled hydriodic acid from the same batch but containing no added hypophosphorous acid; and, with red phosphorous present and absent. No combination of these reagents or conditions prevented or affected the loss of nitrogen at high potassium sulfate concentration.

Measurements have shown that the Friedrich pretreatment does not consume sufficient sulfuric acid to cause the salt/acid ratio to be increased materially. Thus this ratio is not sufficiently increased during the determination to reach that of potassium acid sulfate—a condition which is known to engender nitrogen loss (17).

Results with s-diphenylcarbazone using the former tentative A.O.A.C. method, and with all conditions constant except for a variation of potassium sulfate concentration from 0 to 1500 mg per 1.5 ml of sulfuric acid, showed that acceptable recoveries could be obtained up to the 750 mg level. Postassium sulfate concentrations of 1000 mg or more per 1.5 ml resulted in progressively lower recoveries of nitrogen with increasingly poorer precision.

Fortunately, in the Friedrich determination, the problem of nitrogen loss due to high potassium sulfate concentration need not be serious, since accurate results can be obtained with relatively low potassium sulfate concentrations.

SUMMARY AND CONCLUSION

A group of representative organic compounds containing —NO, —NO_2, =N—N=, and —N=N— linkages was analyzed by the former A.O.A.C. tentative Friedrich microkjeldahl method (333 mg of potassium sulfate per ml of sulfuric acid) and by the same method except that the ratio of potassium sulfate to sulfuric acid was doubled—the approximate ratio and conditions used in the 1949 collaborative study. Satisfactory results were obtained for each of the compounds by the former A.O.A.C. tentative method. Without exception, the increase in potassium sulfate concentration resulted in a significant nitrogen loss and generally in poorer precision. Similar results were obtained with ammonium sulfate.

Studies with s-diphenylcarbazone indicate that complete nitrogen recovery can still be obtained for hydriodic acid pretreated samples by using even less potassium sulfate than the amount called for in the A.O.A.C. former tentative Friedrich method.

On the basis of this study, a high ratio of potassium sulfate to sulfuric acid in the Friedrich nitrogen determination should be avoided.

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MODIFICATION OF THE SCHECHTER METHOD FOR THE DETERMINATION OF METHOXYCHLOR OR DDT IN BIOLOGICAL MATERIALS*

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The presence of residues consisting chiefly of fatty materials has imposed a serious restriction on the application of the Schechter method (1) for the determination of DDT and Methoxychlor in the range of 0.1 to 1.0 parts per million. The difficulties caused by fat are twofold. 1) Interference with the nitration of DDT, and more especially of Methoxychlor;

* Part of the contents of this paper was reported at the meeting of the Federation of American Societies for Experimental Biology, April, 1950, Atlantic City, N. J.

2) The formation of nitrated residues which are difficult to remove, and which may produce yellow "off" colors with the methylate reagent. Two methods have been used with considerable success for the elimination of fat. The method of Schechter (2) consists of separating the DDT from fat by extracting the latter with sulfuric acid. This modification cannot be used for Methoxychlor, which is itself soluble in the acid. The method of Clifford (3) removes fat by enzymatic hydrolysis. In our experience, however, the fat residues are frequently too large, and interfere with the nitration of Methoxychlor. The Fairing (4) method for Methoxychlor achieves small residues by hydrolyzing the sample with ethanolic potassium hydroxide. The resulting dehydrochloride of the insecticide is treated with 85 per cent sulfuric acid to produce a red color. This method is valuable because it permits differentiation from DDT. In our hands, however, it did not prove sufficiently sensitive.

PRINCIPLE OF THE METHOD

The method to be described for Methoxychlor is also applicable to DDT. It consists of saponification of fats by ethanolic potassium hydroxide, with the simultaneous dehydrohalogenation of either compound. Separation of the dehydrochlorides is effected by suitable extraction of the hydrolysate with petroleum ether. These are then nitrated, and red colors developed by treating the nitro compounds with sodium methylate in benzene solution. The colors are stable for extended periods, and are insensitive to moisture. It should be emphasized that the present method does not differentiate among Methoxychlor and either of the isomers of DDT. The maxima of all three absorption curves lie between 490 mμ and 530 mμ.

The sensitivity of the method is of the same order as that of Clifford's modification of the Schechter method. However, the rapid alkaline hydrolysis represents a considerable saving of time, when contrasted to enzymatic hydrolysis which frequently takes two days. Furthermore, the nonsaponifiable residues are much smaller. Mineral hydrolysis produces about 10 mg. residue from six grams of fat, as compared to 50–100 mg. from enzymatic hydrolysis.

REAGENTS

(*a*) *Ethanolic KOH.*—Dissolve 20 grams of reagent KOH in 95 per cent ethanol and make to one liter. On standing the soln may turn yellow from traces of aldehydes in alcohol. This causes no interference.

(*b*) *Petroleum ether, reagent grade, 30°–60° boiling point range.*—Distill once from all glass apparatus. Reject portion boiling above 60°. Even after distillation, technical grade petroleum ether contains materials that are not destroyed by nitration and produce interfering colors.

(*c*) *Nitrating mixture.*—Mix equal volumes of concentrated, reagent grade sulfuric acid and reagent grade fuming nitric acid. Store in refrigerator.

(*d*) *Diethyl ether, reagent grade.*—Distill once from all-glass apparatus. Traces of nonvolatile residue will contribute "off" colors upon the addition of methylate reagent.

(*e*) *Methanol, reagent grade.*

(*f*) *Benzene, reagent grade.*—Distill once from all-glass apparatus. Trace impurities cause interfering colors.

(*g*) *Sodium methylate.*—Prepare as directed in the original Schechter method (1). Calculate weight of sodium on basis of preparing 3.2 *N* soln. This more concentrated methylate will lose any turbidity if stored in a cold place for two weeks before use. The commercially prepared soln is not absolutely colorless, and may cause interference.

METHOD

Lard, butter oil, tissue extracts.—Place weighed samples into 250-ml flasks with standard taper joints. Add a minimum of 50 ml ethanolic KOH for samples under 3 g. For larger samples add 15 ml for each g of sample. The practical limit for the size of the sample is about 6 g. Reflux for 30 min (A). Add one drop of phenolphthalein. If no pink color develops add 15 ml more of KOH and reflux 20 min longer.

Dilute sample with one half its volume of distilled water (B). Cool. Extract once with 100 ml of petroleum ether, and once with 25 ml. Discard the alkaline layer, and wash the combined petroleum ether extracts with 20 ml of 50 per cent aqueous alcohol. Discard the alcohol layer, and pour the petroleum ether extract into a flask suitable for nitration. Add 5 mg of stearic acid in petroleum ether soln to each sample (C). Blow off solvent under a gentle current of air, on the steam bath. Flasks must not be overheated.

Nitrate samples according to the standard Schechter procedure (1). A temperature of 100°C should be reached in 50–60 min. (D). Remove flasks from water bath at once and cool; then dilute with 50 ml cold distilled water. Extract with 50 ml of diethyl ether (E). Discard aqueous layer. Wash ether layer with 10 ml portions of 10% aqueous KOH until both aqueous and ether layers are colorless (F). Wash ether layer once with 15 ml saturated NaCl soln (G). Drain salt soln off carefully. Transfer samples to glass stoppered flasks, filtering thru a plug of glass wool moistened with ether. Rinse separatory funnels with 5 ml of ether; also wash down the filters. Evaporate to dryness on steam bath using a gentle current of air, and continue heating strongly for 20 min. longer (H). Cool.

Add 6 ml of the benzene-methanol mixture, followed by 3 ml of 3.2 *N* $NaOCH_3$. Allow to stand for at least 15 min. (I) before reading in a suitable spectrophotometer (J).

PREPARATION OF STANDARDS

In the 0 to 50 microgram range: Hydrolyse suitable quantities of either Methoxychlor or DDT, including a blank, in 50 ml of ethanolic KOH. Process the blank and standards as described under method, but add 10 mg of stearic acid instead of 5 mg. Plot the relationship between density and concentration. Methoxychlor standards should be read at 530 mμ; p,p'DDT at 520 mμ.

NOTES ON THE METHOD

(A). Two per cent ethanolic KOH is sufficiently concentrated to make a good hydrolysis medium without causing destruction of DDT or Methoxychlor. The actual dehydrohalogenation is completed in 5–10 minutes, but no losses were noted for heating periods up to two hours. Optimum fat hydrolysis is finished after 20 minutes. (Prolonged refluxing did not result in any further decrease in unsaponified residue weight.)

(B). The hydrolysate must be diluted with half its volume of water before extraction. The dehydrochlorides are soluble in 95 per cent ethanol, and their partition between this solvent and petroleum ether is such that their quantitative removal is

not feasible. Greater dilution is not necessary, and enhances emulsion formation from the soaps that are present.

(C). The dehydrochlorides of Methoxychlor and DDT are somewhat volatile. Stearic acid is added at this point to prevent loss of the dehydrochlorides by volatilization during solvent evaporation. If the flasks are permitted to run dry and overheat, losses will occur even with stearic acid present. There is also some evidence to support the view that the presence of this compound prevents "burning up" of the dehydrochlorides during nitration.

(D). The heating period is critical. The nitration must be severe enough to destroy the fat residue. On the other hand, prolonged heating at 100°C progressively destroys the color-producing nitrobenzophenones. The nitration as described gives reproducible colors.

(E). The direct nitration of Methoxychlor gives a variable mixture of a tetranitro derivative analogous to that formed from DDT, and the nitro ketone. The tetranitro derivative of Methoxychlor, which yields a blue color, is extractable with petroleum ether. However, the nitro ketones formed from the dehydrochlorides of DDT and Methoxychlor, which produce red colors, are not soluble in petroleum ether. Therefore, although this solvent extracts less extraneous material, and is generally preferable, it cannot be used in this instance.

(F). Thorough washing with alkali is essential to remove traces of undesirable oxidation and nitration products, which contribute yellow colors on the addition of the methylate reagent. Three washes are usually sufficient. An increased number of washes may be used, however, if necessary. The nitro ketones are insoluble in 10 per cent KOH, and no losses will result.

(G). The salt wash removes traces of alkali which will cause losses during the subsequent evaporation and heating of the samples.

(H). As previously noted by Clifford (3), this extended heating period destroys traces of contaminating material carried over, and reduces any yellowish "off" colors in the final step.

(I). This particular concentration of sodium methylate has been chosen because it produces maximum color intensity. The red color of Methoxychlor develops at once, but at least 15 minutes are needed to obtain the maximum intensity of the DDT color. Once developed, the colors are unaffected by moisture and are otherwise stable. A sample of methoxychlor diluted with 0.5 ml of water showed only the drop in color intensity expected from the increase in volume of the solution. Samples reread after three hours showed no fading.

(J). We have used both a Bausch and Lomb visual spectrophotometer and a neutral wedge photometer with 5 cm cells. Somewhat greater sensitivity can be obtained with a Beckman photoelectric instrument.

RESULTS

Figure 1 shows representative absorption curves for Methoxychlor, o,pDDT, p,p' DDT, and a blank. These curves were obtained with a Beckman photo-electric spectrophotometer, on colors developed from 50-microgram quantities of the compounds. It is evident from the location of the absorption maxima that quantitative differentiation in mixtures of the compounds would be difficult. The blank gives measurable absorption in the ultraviolet due to the presence of stearic acid residue. However, the stearic acid contributes practically nothing in that visible range where the compounds show maximum absorption.

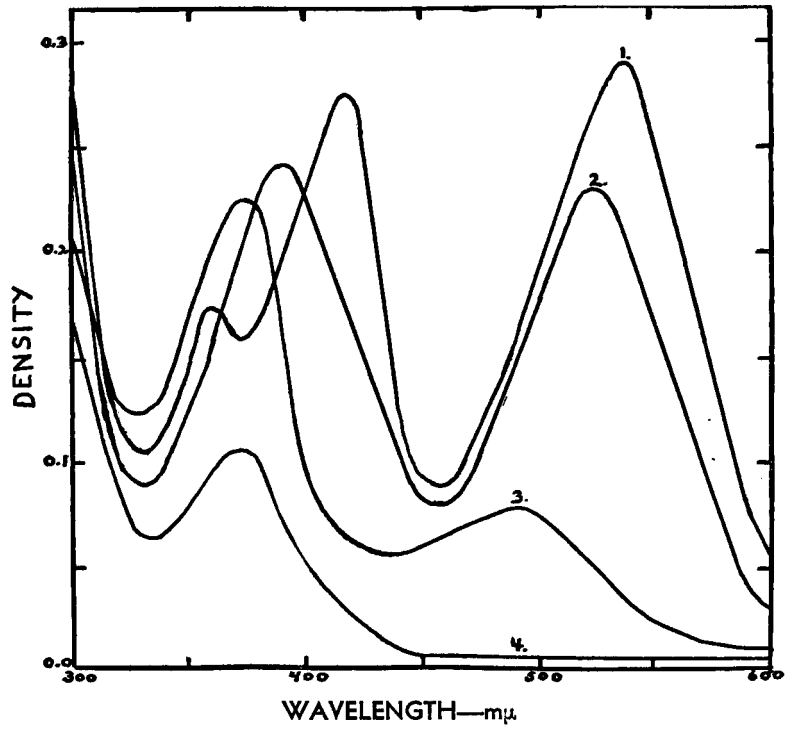

FIG. 1.—Absorption Curves.

1. Methoxychlor—50 micrograms
2. p,p'DDT—50 micrograms
3. o,p'DDT—50 micrograms
4. 10 mg. stearic acid

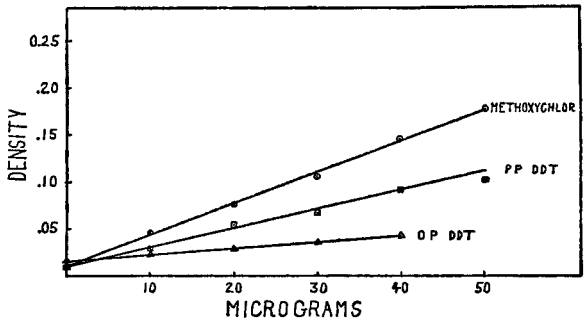

FIG. 2.—Relation between density of the colors developed for Methoxychlor, p,p'DDT, o,p'DDT and the respective concentrations of the compounds.

Figure 2 shows the relationship between the concentration of the compounds and the optical density of the colors developed from them, read at the point of maximum absorption for each. Note that the slope is greatest for Methoxychlor and least for opDDT.

TABLE 1.—*Recovery of added DDT and Methoxychlor*

SUBSTANCE		DDT		METHOXYCHLOR	
	AMOUNT	ADDED	FOUND	ADDED	FOUND
	gms	*μg*	*μg*	*μg*	*μg*
Lard	3.0	25	27	10	11
	3.0	50	51	—	—
	2.5	—	—	50	49
	2.0	40	42	20	22
Milk	100	13	12	10	9.5
	100	43	45	17	17
	100	50	52	45	46
Butter	3.8	40	40	40	41
	6.0	5.0	5.2	—	—
	6.0	20	23	—	—
Rat Fat	1.8	—	—	20	18
	1.8	—	—	40	41

Table 1 contains data on the recovery of known amounts of DDT and Methoxychlor added to a variety of fatty materials. The limit of quantitative sensitivity of the method is about 5 micrograms contained in 5–6 grams of extracted fat. For milk the range is about 5 micrograms in 100 ml. Interference develops if the nonsaponifiable residue from samples weighing over six grams is nitrated.

TABLE 2.—*Comparative results of DDT analyses of milk and fat*

	SCHECHTER-HALLER ENZYMATIC HYDROLYSIS	DEHYDROHALOGENATION ALKALINE HYDROLYSIS
	p.p.m. DDT	*p.p.m. DDT*
Milk	0.12	0.12
	0.10	0.09
	0.17	0.19
	0.10	0.18
	0.16	0.15
	0.14	0.10
	0.11	0.14
Fat	4.9	5.7
	3.6	3.4
	1.9	2.8

Since DDT is determined as the dehydrochloride, the possibility arose that dehydrochloride occurring as a possible metabolite in the tissues of animals exposed to DDT, might give rise to erroneously high results. Table 2 contains comparative results obtained by our method, and by Clifford's method, which determines DDT alone. The agreement between them is satisfactory.

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A SPECTROPHOTOMETRIC METHOD FOR THE QUANTITATIVE ESTIMATION OF TECHNICAL CHLORDANE*

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Since the insecticidal properties of DDT were first recognized and reported, a wide variety of other chlorinated compounds have been, and are still being, proposed as substitutes for, or improvements on, that compound. Among the recently developed synthetic organic insecticides, technical chlordane has shown real promsie in the control of some insects of public health and agricultural importance. The widespread use of technical chlordane has, of course, posed many analytical problems related to its determination.

Although analytical methods based on total chlorine (1), biological response (2), and on two different color reactions (3, 4) were available, they were not considered applicable to the determination of technical chlordane in biological tissues or spray residues, since they all lacked either sensitivity or specificity. Therefore, a search was made for a method more suitable for the *specific* estimation of the relatively small quantities of technical chlordane which might occur in biological tissues or spray residues. The following method was finally developed:

METHOD

APPARATUS

Beckman Spectrophotometer.

Chromatographic tubes—40 mm. O.D. × 200 mm. long.

Glass tamping rod—Diam. of flat portion somewhat less than diameter of chromatographic tube.

* The data are taken from the dissertation submitted by Bernard Davidow to the Faculty of the Graduate School, Georgetown University, 1950, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

REAGENTS

(*a*) *Technical chlordane*—Reference grade,[1] and redistilled technical grades.[2]

(*b*) *Silica gel.*[3]

(*c*) *Normal Hexane Commercial*[4].—Purify by passage through silica gel column (500 g silica gel will yield 500 ml purified hexane).

(*d*) *Celite* 545.[5]

(*e*) *Fuming sulfuric acid (15% SO_3).*

(*f*) *Normal methanolic potassium hydroxide.*

(*g*) *Diethanolamine.*

(*h*) *Diethanolamine-potassium hydroxide reagent*—2 volumes reagent (**f**) plus 1 volume reagent (**g**).

PREPARATION OF STANDARDS

Prepare a 1 mg/ml standard soln of reference, or redistilled technical grade chlordane in hexane. To 5 test tubes calibrated at 5 ml add 0.0, 0.1, 0.2, 0.4, and 0.6 ml, respectively, of the standard soln. Add to each 3 ml of the diethanolamine-potassium hydroxide reagent (**h**), and heat in a boiling water bath for 30 min. Cool to room temp. in cold water and allow to stand for 15 min. Add methanol to the 5 ml mark, shake, and then transfer solns to the absorption cells. Note the absorbency of the standards at 521 millimicrons and construct a standard concentration curve (absorption obeys Beer's law).

DETERMINATION

Treat a sample containing between 50 and 700 mmg of technical chlordane as follows:

(1) *Biological tissues.*—Place weighed tissue in a mortar, add anhydrous sodium sulfate equal to ca 3 times the weight of the tissue. Grind to a coarse dry powder to insure dehydration and rupturing of the cells. Transfer to a soxhlet and carry out extraction with ether for a period corresponding to at least 10 syphonings of the apparatus. Evaporate the ether by gentle heat and dissolve the residue in 15 ml of hexane (**c**).

Separate the insecticide from interfering substances by chromatographing thru a column (5) prepared as follows: Place 30 g of celite (**d**) in a mortar, and add 9 ml of concentrated sulfuric acid and 9 ml of fuming sulfuric acid (**e**). Triturate until a homogeneous, slightly damp powder is obtained. Add 100 ml of hexane (**c**) in 4 portions and triturate the mass to a smooth slurry. Plug the constricted end of a chromatographic tube with glass wool and attach a short piece of rubber tubing fitted with a closed screw clamp. Pour ca 25 ml of hexane (**c**) into the tube and, with a porcelain or glass spoon, add the slurry in several portions, packing it down with the tamping rod after each addition. (The finished column must appear homogeneous and be of uniform firmness across the diameter. A little experience will show how firmly the column must be packed in order to obtain an optimum flow rate: ca 120 drops per min.) After preparation of the column, a little free hexane should stand above it. (If more than a few ml remain, drain off until the height is only a few mm above the celite.) Now pipette the hexane soln of the sample onto the column, disturbing the surface as little as possible. Remove the tubing and allow the soln to pass into the column. Start collecting the percolate immediately after the soln has completely passed into the column. Rinse the flask or beaker, and the sides of the tube, with about 25 ml of hexane (**c**) added in 3–4 portions, allowing each rinse

[1] Velsicol Corporation product used.
[2] Julius Hyman and Company product used.
[3] Davison Chemical Corporation product used.
[4] Phillips Petroleum Company product used.
[5] Johns Manville Company product used.

to sink into the column before adding the next one. After the final rinse has passed into the column, fill the tube with hexane (c) and collect about 200 ml of the eluate. (The column is designed to retain about 5 g of fat.)

Concentrate the eluate by gentle heat and a current of air, to about 10 ml. Transfer in 2 portions to a test tube calibrated at 5 ml, and continue concentration of the chlordane soln to a volume of ca 1 ml. Add 3 ml of the diethanolamine-potassium hydroxide reagent (h), and heat the mixture in a boiling water bath for 30 min. Cool the tube to room temp. by immersing in cold water and allow to stand for 15 min. Adjust the volume to 5 ml with methanol, and determine the absorbency of the soln at 521 millimicrons. Calculate the concentration of chlordane by comparing the absorbency of the unknown with that of a standard concentration curve.

(2) *Spray residues.*—Place a weighed amount of sample in a 2 liter wide mouth bottle containing a measured volume of benzene (between 250 and 500 ml, depending on the crop under analysis. Shake the mixture for 5 min. and drain off the benzene as completely as possible (note volume). To the benzene soln add 10 g of a decolorizing mixture containing 10 parts Nuchar,[6] 5 parts Filtercel,[6] 5 parts Attapulgus clay,[7] 10 parts silica gel, and 5 parts anhydrous sodium sulfate. Shake the soln vigorously for 5 min. with the decolorizing mixture and filter on a folded paper. (This operation dehydrates the benzene and also removes the natural plant pigments from the benzene soln.) Concentrate the colorless benzene filtrate (note volume), by gentle heat and a current of air, to 10 ml, and follow the procedure described under "Biological Tissues" beginning with "Transfer in 2 portions to a test tube calibrated at 5 ml." Calculate the concentration of chlordane from the absorbency of the colored reaction product, and the weight of the sample represented in the aliquot.

(3) *Commercial concentrates.*—The determination of technical chlordane in commercial concentrates is performed by preparing known dilutions in hexane (0.5 mg technical chlordane per ml) and developing the colored reaction product in the manner described under "Preparation of Standards." The concentration of technical chlordane can be calculated from the absorbency of the colored reaction product, and the weight of the sample represented in the aliquot.

RESULTS AND DISCUSSION

Technical chlordane[8] is the commercial product containing 60 to 75 per cent of chlordane (see Table 1) together with 40 per cent of other related compounds normally resulting from the manufacturing processes. All of its ingredients are considered active under the Insecticide Act (6).

From the reactions involved in the synthesis of chlordane, one may reasonably postulate a number of compounds as possible constituents of the technical product. Several of these have been isolated from the material known as technical chlordane (7) (See Table 1) as crystalline compounds by chromatographic technique. Components other than those listed in Table 1 are also present in technical chlordane. They are believed to consist principally of chlorinated dicyclopentadienes of varying chlorine content. However, not enough information is yet available for defining their structures.

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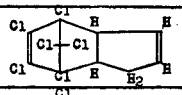
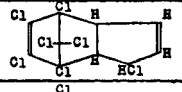
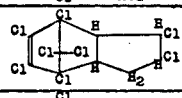
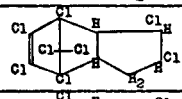
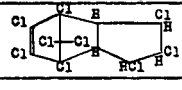
[6] West Virginia Pulp and Paper Company product used.
[7] Attapulgus Clay Company product used.
[8] Technical chlordane is sold under the trade-names Velsicol 1068 and Octa-Klor.

TABLE 1.—*Compensated isolated from technical chlordane*

Empirical Formula	Structural Formula	Common Name	Melting Point °C.	Insecticidal Activity LD_{50} for Adult Milkweed Bug
$C_{10}H_6Cl_6$		"237"	155°-185°	1000 Micrograms
$C_{10}H_5Cl_7$		Hepta-Klor	95°	31 ± 1 Micrograms
α Chlordane $C_{10}H_6Cl_8$		Alpha-Chlordane	102°-104°	459 ± 14 Micrograms
β Chlordane $C_{10}H_6Cl_8$		Beta-Chlordane	104°-105°	44 Micrograms
$C_{10}H_5Cl_9$		Trichloro 237	122°-124°	Nontoxic
Technical Chlordane	Principally mixture above components	Octa-Klor	Liquid	143 ± 2 Micrograms

When the proposed method was tested upon different preparations of technical chlordane it was found that there were variations in the absorption spectra of their colored reaction products. This no doubt was due to the fact that each of the products examined consisted of a somewhat different mixture of isomers and related compounds. The determination of technical chlordane, nevertheless, may be accomplished without resolution of the individual components, since the colored reaction products have overlapping absorption bands (8) (See Figure 1). Thus at an intersection point (521 millimicrons with the spectrophotometer employed using a slit width of .04 mm) the absorbency of mixtures of technical chlordane is unaffected by variations in the relative proportions of each product. Absorbency readings at this point are influenced only by variation in technical chlordane concentration in the direct proportion required by Beer's law. Therefore, in order to determine technical chlordane it is necessary only to prepare a solution of appropriate concentration, and determine the absorbency of the colored reaction product at the intersection point. From the ratio of the value obtained, as compared to a standard, the technical chlordane content of a mixture can be calculated. Table 2 summarizes the technical chlordane content of various batches of commercial concentrates.

Specificity of the reaction.—A number of insecticides and structurally related compounds were submitted to the potassium hydroxide-diethanolamine treatment in order to determine the specificity of the reaction.

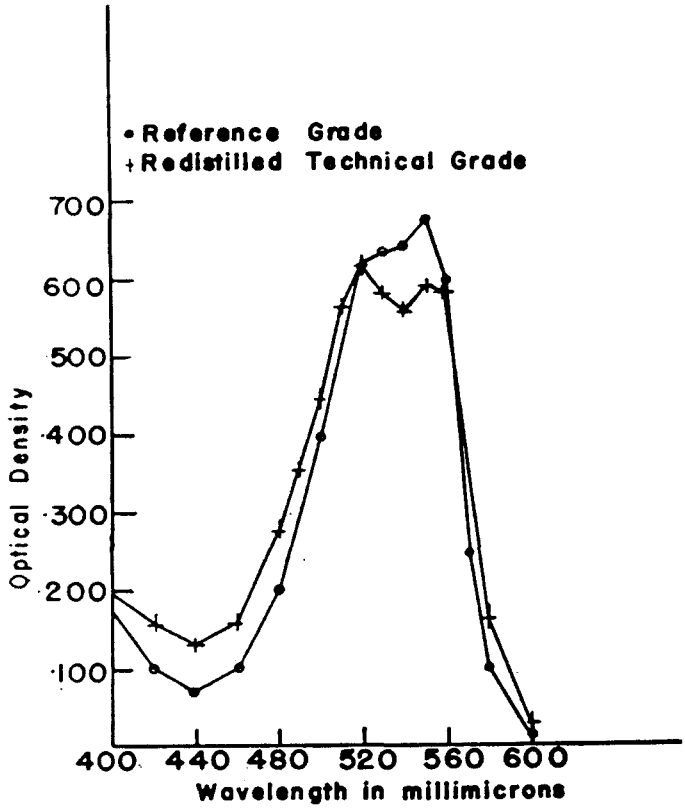


FIG. 1.—Absorption spectra of the colored reaction product of reference grade and of redistilled technical chlordane. Concentration, 0.5 mg/5 ml.

TABLE 2.—*Technical chlordane content of various batches of commercial concentrates*

GRADE	DATE OF MFR.	PER CENT TECH. CHLORDANE
Technical	April 1946	74
Agricultural	October 1947	94
Refined	October 1947	100
Technical	February 1948	98
Standards:		
Reference	April 1946	100
Redist. Tech.	October 1947	100

Approximately 25 mg of each substance were used in the test. The findings are recorded in Table 3. Also, it was found that chlorinated insecticides such as DDT, benzene hexachloride, toxaphene, and TDE, in amount 20 times the concentration of technical chlordane, did not interfere with a quantitative estimation of technical chlordane. It will be noted that of

TABLE 3.—*Specificity of reaction*

Compounds Isolated from Technical Chlordane	
1. Compound 237	Colorless
2. Heptaklor	Blue-red color
3. Alpha Chlordane	Blue-red color
4. Beta Chlordane	Blue-red color
5. Trichloro 237	Red color
Other Insecticides and Structurally Related Compounds	
6. Aldrin	Colorless
7. Alpha Benzene Hexachloride	Colorless
8. Beta Benzene Hexachloride	Colorless
9. Gamma Benzene Hexachloride	Colorless
10. Delta Benzene Hexachloride	Colorless
11. Technical Benzene Hexachloride	Colorless
12. Borneol	Colorless
13. p,p' DDT	Colorless
14. o,p DDT	Colorless
15. Methoxychlor	Colorless
16. Dieldrin	Colorless
17. 3,4 dinitro anisole	Orange color
18. Glyodine	Colorless
19. HETP	Colorless
20. Hexachlorocyclopentadiene	Yellow Color
21. N-propyl isome	Yellow Color
22. Octacide	Colorless
23. Parathion	Yellow Color
24. Crude Piperonyl Butoxide	Dirty Brown Color
25. Pyrethrum	Light Orange Color
26. Rotenone	Red Color
27. TDE	Colorless
28. Thanite	Colorless
29. Toxaphene	Colorless
30. Tryptophan	Colorless

all the compounds tested, only rotenone gave a color which might be confused with the colored reaction product of chlordane. However, there are 3 methods by which technical chlordane can readily be distinguished from rotenone. First, the colored reaction product of each has a characteristic absorption spectrum. Second, the colored reaction product of technical chlordane is not extracted by ether, benzene, carbon tetrachloride, hexane, or chloroform; on the other hand, the colored reaction

product of rotenone is extracted by all the above mentioned solvents. Finally, rotenone is separated from technical chlordane by the acid-celite column described under the determination of technical chlordane in biological tissues.

From the data presented in Table 3 it appears that the color reaction is dependent upon the presence of a chlorine addition or substitution in the cyclopentene ring of the 3a,4,7,7a-tetrahydro-4,7-methano-indene.

RECOVERIES

To test the performance of the method with respect to biological tissues and spray residues, technical chlordane was added to extracts of such biological tissues as rat fat, liver, and kidney, and to benzene extracts of cabbage and pears. The results are tabulated in Table 4.

TABLE 4.—*Recovery of technical chlordane added to extracts of biological tissues, cabbage, and pears*

SAMPLE	WEIGHT	MICROGRAMS TECHNICAL CHLORDANE ADDED	% RECOVERY
Fat	2.24	500	90*
Fat	3.97	250	80*
Kidney	4.00	500	88*
Kidney	4.00	250	89*
Liver	6.00	500	87*
Liver	6.00	250	90*
Cabbage	100.00	800	85
Pears	500.00	400	104
			Av. 89

* Compared with a standard which had been chromatographed on an acid-celite column.

In preliminary experiments it had been noted that certain oxygenated compounds, such as acetone and rancid fat, interfered with the formation of the colored reaction product. These compounds, however, can be separated from technical chlordane by the use of the acid-celite column.

The method as described does not differentiate the components of technical chlordane. At its present stage of development, the method is applicable to the determination of technical grades of chlordane, and to the estimation of alpha chlordane, beta chlordane, heptaklor, and trichloro 237, when only one is present (See Figs. 2 and 3, and Table 5. The absorption spectra of heptaklor and beta chlordane are similar to that of alpha chlordane). For the estimation of heptaklor, alpha chlordane, and beta chlordane (when only one is present), the optimum wave length is 560 millimicrons; for the trichloro 237 compound, the optimum wave length is 515 millimicrons.

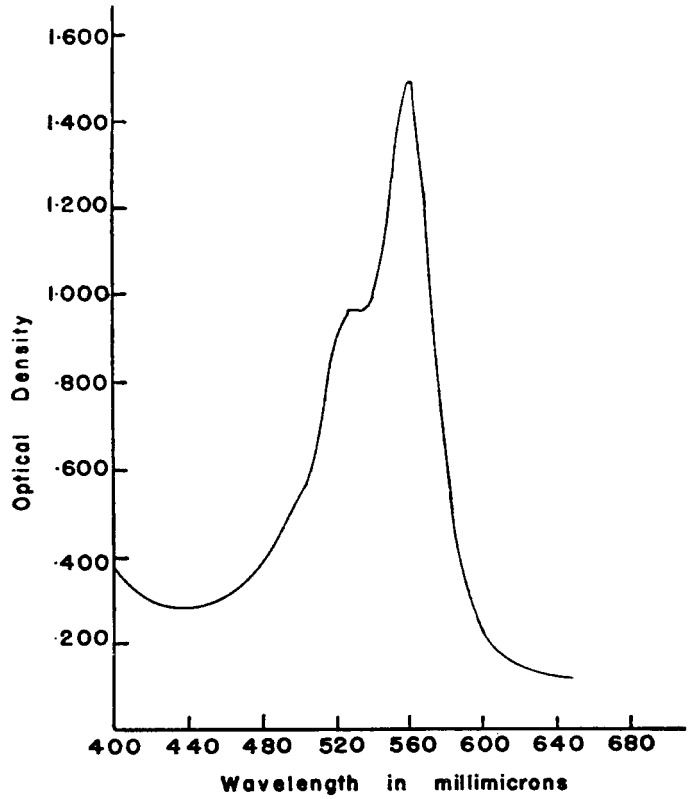


FIG. 2.—Absorption spectrum of the colored reaction product of alpha chlordane. Concentration, 1.065 mg/5 ml.

An analysis of the recovery data reveals an average recovery of 89 per cent, with an average deviation of 5 per cent. The source of error is mainly due to the volatility of technical chlordane. (When standard solutions of the insecticide were concentrated from a 200 ml volume of hexane, recoveries were only 90%.) The method is empirical in nature; therefore the analyses should be performed under the identical conditions used for the preparation of standard curves. In this manner as little as 50 micrograms of technical chlordane per total sample may be determined in extracts of biological tissues, and in such crops as cabbage and pears.

SUMMARY

An analytical procedure has been described in which technical chlordane and the known pure isomers of chlordane, as well as most of the other

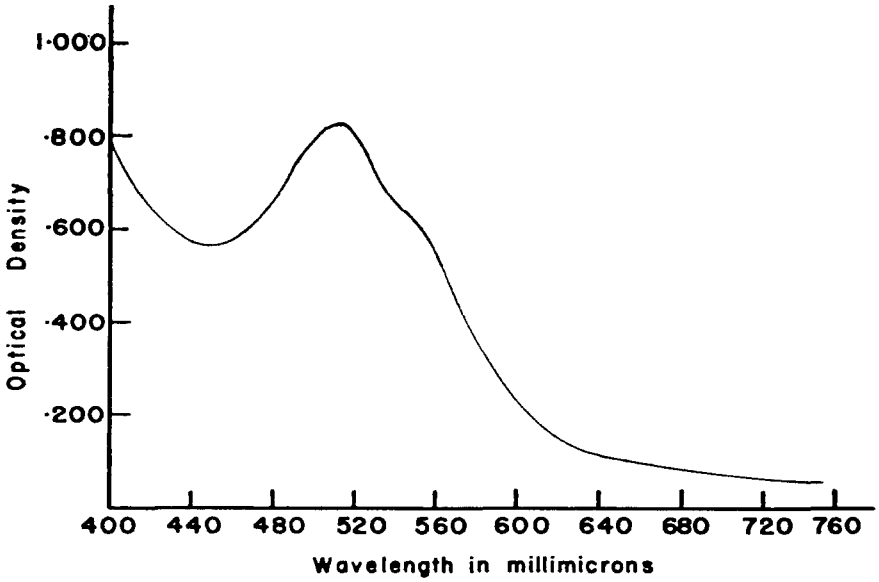


FIG. 3.—Absorption spectrum of the colored reaction product of trichloro 237. Concentration, 1 mg/5 ml.

TABLE 5.—*Molecular extinction of compounds isolated from technical chlordane*

COMPOUND	MOLECULAR EXTINCTION	WAVE LENGTH
Alpha Chlordane	2870	560
Beta Chlordane	950	560
Heptaklor	9700	560
Trichloro 237	1860	515
Compound 237	None at 515 and 560	

components isolated from technical chlordane, give a colored reaction product.

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IDENTIFICATION OF INSECT FRAGMENTS IN SPICES AND OTHER FOOD MATERIALS UTILIZING A DIFFERENTIAL STAIN

By SALVATORE J. SICA and ROBERT A. NANZ (Foster D. Snell. Inc., New York 11, N. Y.)

The complete separation of insect fragments from the food materials is impossible by mechanical means, making the determination of the amount of insect fragments in the food or food debris very difficult and time consuming. This is true with such cereal products as rye or whole wheat flour, and especially with ground spices where there is a close resemblance in color and form between the insect material and the spice particles. Since, in these foods, the insect fragments are ground to the same size as the food itself, sieving cannot be used to effect a complete separation of the fragments for identification. It therefore becomes necessary to resort to a flotation method based on the fact that the insect particles have a greater affinity for oil than do the food particles. The A.O.A.C. flotation method for white flour (1) has been modified by Harris and Knudsen (2), who also studied the method of Walker and Dalby (3) which incorporates an acidified acetone digestion. Staining is not employed in any of these methods, however. A staining technic has been reported by Gier, Wilbur, and Miller (4)for use in white flour determinations. Dye is added after pancreatin or hydrochloric acid digestion and trapping off in a Wildman trap-flask or a separatory funnel. This procedure will stain the cereal product preferentially to a shade of blue, while the insect fragments stain green. The method does not include the use of acetone for defatting the particles.

Acetone acts as an effective solvent for the oils in food materials, allowing materials like spices to be wetted; further it probably frees foreign matters, such as rodent hairs, for separation. In the method presented below we have combined the acetone modification with the staining procedure, which was then further adjusted to provide a more specific differentiation. The color differentiation that results makes it much easier to count the insect fragments. This method has been tested on rye flour, whole wheat flour, mustard flour, ground coriander, and ground paprika.

The method as given by Walker and Dalby in Cereal Chemistry (3) comprises the first phase of the method:

PROCEDURE

Place a 10-g sample of ground spice (or other food material) in a dry 1-liter Florence flask. Add 200 ml of acetone and swirl flask until solvent and spice are mixed. Add 380 ml of distilled or boiled water, 20 ml of concd hydrochloric acid and 20 ml of light mineral oil. Place flask under reflux condenser and boil gently for 20 min. Cool to 25°C. and add 50 ml of gasoline or benzine. Decant into separatory funnel. After settling, open clamp and discard heavy particles.

The second step is an adaptation of the Gier *et al.* method (4).

Add 0.1% sodium bicarbonate to neutralize to *p*H 7.0. Check with *p*H paper. Trap down to smallest practicable volume of oil layer plus water soln. Pour trapped layer into small beaker. Add 0.5% of the stock soln of Azure-1.

Place in oven or water bath at 40°C. (104°F.) for 30–35 min. Pour the stained material into a separatory funnel. Wash beaker with distilled water, add washings to fluid in the funnel. Add petroleum ether, shake well to assure complete reflotation of all particles. Allow 5 min. for particles to float, drain off most of the water. Wash once or twice with 100 ml. distilled water, to remove excess stain. Filter on lined paper using suction. Examine at 36 power.

EXPERIMENTAL WORK

In the first experiment, insect fragments were purposely placed in plain water as a test solution. The complete flotation technic was followed, using 0.5% of the dye solution (1% Azure-1 in methyl alcohol). This is added after the digested material in solution is neutralized and the oil layer trapped off.

This was repeated using certain spices. The dye was allowed to penetrate the defatted spices. In these experiments, records were made of the type of insect fragments added to the ground spice sample for recovery, noting especially their color and shape.

RESULTS

As shown in Table 1, the clear-cut distinction between colors can aid greatly in the counting of insect fragments. Analysts had worked with

TABLE 1.—*Recovered insect fragments placed in natural ground spices and certain cereal flours*

TEST MATERIAL	INSECT FRAGMENTS ADDED PER 10 G	INSECT FRAGMENTS RECOVERED PER 10 G	COLOR OF FOOD MATERIAL	COLOR OF INSECTS FRAGMENTS
Water	6	5	—	Unchanged
Mustard Flour	None	None	Bran and Mustard: Deep Blue	—
Mustard Flour	7	6	Blue	Unchanged
Mustard Flour	5	5	Blue	Unchanged
Coriander, Ground	7 (2 white larvae, 1 black antenna, 4 brown leg sections)	7	Blue	Unchanged
Whole Wheat Flour	None	None	Blue	—
Rye Flour	4	4	Blue	Unchanged

greater quantities of dye at first, as suggested by Gier, *et al.* (4), but the difference in colors was not satisfactory, the particles all staining in variable intensity. Therefore the amount of dye was changed to reach a point where a difference in color was quite noticeable. After such adjustment, it was found that the insect fragments differed greatly in color intensity, being relatively unchanged, not stained as are the food particles. Several rechecks were made to verify the results. Mustard flour, on first examination using the heavier stain, appeared to contain very great amounts of insect fragments, because of their resemblance to the bran particles. The lighter staining technic helped greatly here. Coriander, in powder form, also contains particles which resemble insect fragments when not differentially stained. With our method, the ground spice particles stain noticeably, while the insect fragments do not take up the dye. Whole wheat flour, with bran particles resembling insect fragments, was successfully examined when the bran was stained preferentially leaving the insect fragments unchanged. The proposed method does not stain the insect exoskeleton or any worm forms (larvae), either because of the smaller quantity of dye, or because of the action of the acetone on the insect fragments.

SUMMARY

A dye, Azure-1 in 1% methyl alcohol solution, used in small quantities, was added as a step in the acetone flotation method for the determination of insect fragments in foods. It stains food particles preferentially, leaving unchanged the insect fragments present. The difference is quite distinct between the appearance of the two types of particles, the insect fragments being in their natural color, whereas the food or spice particles are dyed. The stain is picked up by mustard flour, whole wheat flour, and ground coriander. Although red particles, such as paprika, do not pick up the dye evenly, but assume a greenish blue coloration, the dye has no effect on insect fragments, which can be distinguished clearly.

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IDENTIFICATION OF INSECT CONTAMINANTS OF FOODS BY THE MICRO-MORPHOLOGY OF THE INSECT FRAGMENTS

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I. INTRODUCTION

Methods for the determination of insect parts in food products may be divided into two parts: (1) isolation and/or concentration of the insect debris from food materials, and (2) microscopic identification and counting of the insect material present. The sixth edition of *Methods of Analysis* of the A.O.A.C. (1) contains a chapter on methods for the isolation of extraneous materials, including insect filth, from foods. Harris (2) published a bibliography of 157 references to such methods. The present paper is concerned with phase two of the methods—microscopic examination for insect parts.

This actual determination of insect[1] material is based upon a knowledge of the morphological and histological characteristics of both the comminuted food material and the insect fragments. The problem of identification of various plant tissues has been discussed by several authors. Youngken (3) describes a majority of the drug plants. Winton (4) gives the histology of food raw materials. The classic volume on insect micromorphology is that by Snodgrass (5) who describes homologous insect structures in the various insect body regions and organ systems. More recently, four papers have appeared which were a direct result of regulatory work on food sanitation, and which dealt with specific problems of food analysis for insect and rodent filth.

Essig and Michelbacher (6) figured the various anatomical parts of the corn earworm, tomato pinworm, and potato tuber moth as they occur in tomato products. Nelson (7) discussed the structural peculiarities of some adult and immature insects responsible for the adulteration of foods. The general problem of insects and insect parts as filth in foods is treated in Microanalysis of Food & Drug Products (8). Harris and Nicholson (9) briefly discussed the problem of insect fragment identification as related to cereal products. The present paper appears to be the first comprehensive discussion of the identification of insect fragments as such, and of the determination of the types of insects from which they were derived.

II. INSECT FRAGMENTS VERSUS PARTICLES OF FOOD

The methods currently used for the isolation and microscopic determination of insect fragments serve to isolate only those fragments which result from the shattering of the insect exoskeleton or outer shell. Except

[1] The term "insect" is used in its popular sense to include arachnida and insecta.

for breathing trachea (Figure 24 F) and maggot mouthhooks (Figs. 21 and 22), both of which are morphologically part of the chitinized cuticula, the internal organs are either not isolated or not visible by the microscopic examination employed. Consequently, the study of insect fragment identification is a study of the insect cuticle, and how it differs from the plant structures which form the raw materials from which we obtain our food products.

Some types of plant tissue fragments are readily separated from insect parts. The starch-bearing parenchyma is composed of cells with thin walls hidden by the masses of starch. This tissue, which includes the starchy endosperm of the cereal foods such as corn and wheat, breaks into large, irregular, chunky, pearly white pieces which bear little resemblance to insect parts. Similarly the meristematic tissues of the growing tips or seed embryo, and the conducting tissues, are readily distinguished from pieces of insects. The former is composed of compact thin-walled cells which, upon being comminuted, do not fracture into thin plates as does the insect cuticula, but rather into more rounded chunks. The conducting tissues and strengthening fibers of plants, with their elongate and readily visible cell walls, bear no resemblance to insect parts.

It is the epidermal layers which are troublesome and warrant further attention. These layers cover the surface of all parts of plants that are not protected with bark. The layer usually is one cell thick. Over the surface of the outer epidermal wall of the aerial parts of most plants there is deposited a waxy substance termed cutin, which forms the cuticle. The cuticle is thin and not visible at the magnifications used for insect fragment counts, but is of importance because its oleophilic properties result in its being wetted by the oils used in filth extractions, and extracted, along with adhering plant tissues, in the oil strata carrying the insect parts. (When acting as a seed coat the epidermis is termed the spermoderm, and in this role it sometimes bears a resemblance to the insect cuticula.)

Chaff present in commercially prepared grains and, to a lesser extent, in milled cereal foods, is a collective term for various flower parts that are mainly modified leaves with compact layers of thin cellular tissues, whose yellow to brown color gives them a resemblance to insect fragments. They can be recognized on the basis of their characteristic cellular pattern.

Thus, while many plant structures bear a superficial resemblance to certain insect fragments, a close examination will reveal the cellular structure of the plant fragments. However, the seed coats from certain weed seeds (*e.g.*, dock, cockle, and mustard) are so heavily pigmented that the cell walls are obscure. Moreover, the surface pattern and shape may bear some resemblance to insect cuticle. For example, mustard spermoderm is smooth and rounded, and so may appear to be a portion of the insect pronotum. The angular corners of dock can contribute fragments that have insect-like sutures. The roughened spermoderm from corn cockle

may break into pieces that appear to be from the toothed thorax of the sawtoothed grain bettle. (For description of other weed seeds, see Pammel and King (10).) Thus no one character can be used alone without applying some judgment as to its diagnostic value.

As shown in Figure 1, the cell pattern may take on many forms, while the insect cuticle (Fig. 2) is composed of a secreted, smooth or roughened, lacquer-like layer, often interlocking at the sutures or joints—*never cellular*. In addition to this negative approach, the insect cuticula bears many characteristic structures visible at the relatively low magnifications of 20×–40×.

III. CHARACTERISTICS OF INSECT FRAGMENTS

The insect integument, or exoskeleton, consists of an outer secreted cuticle and an inner layer of epidermal cells. The cuticle is a non-living, clear or pigmented coating composed of proteins and insoluble carbohydrates some of which are resistant to strong acids, and some to strong alkali. As is the case in most animal tissues, the cell walls of the underlying cellular layer are visible only after being stained and examined under the compound microscope. As extracted from food products, this inner layer is collapsed against the outer cuticula and passes completely unnoticed. In contrast, because of the thickened cellulose cell walls, the basic cell structure of plant tissues is plainly visible. Throughout the plant, the cell unit is the basic structure although in storage organs, such as the starchy endosperm of grains, the walls are thinly compressed between the starch grains. Thus, not only are there certain characteristics by which insect fragments can be distinguished; but, as discussed below, the characteristics are in contrast to those found in plant tissues.

A. *Absence of cell structure and*
B. *Translucence*

These two characters have been discussed in the text and figures. (The translucence, especially when mounted in oil or glycerol, may be sparking clear.) When making insect fragments counts, they probably serve as the criteria which commonly arrest the attention of the operator, so that he checks for accompanying confirmatory characters. Both of the characters are due to the fact that the insect cuticle is a secreted layer which flows into position and hardens into a lacquer-like structure. (Fig. 5, B).

C. *Composed of interlocking or hinged plates (Figs. 2, 4)*

A complete understanding of this point involves the entire field of insect morphology, which is treated fully by Snodgrass (5). For the present purpose it should suffice to note certain basic points.

Insects are divided, anterior-posteriorly, into three body regions: head,

thorax, and abdomen. In addition to these three primary body divisions, the thorax and abdomen are further segmented. This segmentation is most apparent in certain larvae and in the abdominal plates of most adult insects. The thorax is divided into pro-, meso-, and metathoracic regions, each highly modified as leg- and wing-bearing mechanisms. However, in spite of the individual characteristics of each insect and, in fact, of each body segment, the thoracic and abdominal regions are constructed of certain basic homologous structures. Each segment has an uppermost plate (dorsum), a more or less membranous side region which often is considerably shrunken or distorted (pleura), and a tough ventral plate (venter). In the head, these regions are modified beyond recognition; they are considerably convoluted in the thorax; but they appear in what is believed to be their basic form in the abdomen. The dorsum of the prothorax consists of a relatively thick, simple, armor-like shield called the pronotum. In the two wing-bearing segments each notum contains indentations, sutures, pits, and ridges which serve as points of attachment for the wing muscles. The legs are attached in the pleural regions, each point of attachment being surrounded by, and composed of, rigid plates to which muscles are attached. The ventrum also is modified for muscle attachment. These features, when considered in conjunction with the development, degeneration, flattening, elongation, compression, or expansion which is characteristic of each individual species, produce a variable assortment of interlocking plates which by their own complex form serve to distinguish insect fragments.

D. *Setae, seta bases, and spines; and*
E. *Surface pattern*

Under these two headings may be classified the most commonly encountered diagnostic characters by which a positive identification of insect material can be accomplished. Although these features have been mentioned earlier, they are of sufficient importance to warrant further attention. (See Figs. 2, 5, 10, 15A, D, E, F.)

Although the gross structural detail of legs, wings, mandibles, and other discrete body parts serve to identify them, in the routine counting of highly comminuted fragments, such parts usually are not whole: in fact they have been reduced to portions of small pieces of the various body regions. Pieces of abdomen, pupae, or elytra may show none of the natural shape of these parts, and the presence of fine spines, seta bases, small pits, and irregular roughening must serve as diagnostic characteristics. (See Figs 3A and 5.) The use of surface pattern is particularly striking on the confused flour beetle elytra fragment shown in Figure 3A, where it is seen that even the small piece delineated by the area a b c would be recognized as insect material.

IV. INSECT FRAGMENTS AS A MEANS OF DIFFERENTIATING TYPES OF INSECTS CONTAMINATING FOODS[2]

It is convenient to consider the common food-contaminating types in three groups: Pests of the growing plant, storage pests, and other types of special significance. The grouping is more convenient than exact and obviously there is a great deal of overlapping. Ants, for example fall in either of the first two categories. Some grain beetles begin their work on the growing plant and continue thru into storage. Fragments of caterpillars may come from field infesting species (corn earworm in tomatoes) or from storage pests (Angoumois grain moth in wheat).

A. *Field Contamination*

The destructive activity of many groups of insects is confined exclusively to the growing plant. Leafhoppers, aphids, scale insects, thrips, and true bugs have sucking mouthparts and can feed only on green succulent plants.

1. Fragments of leafhoppers (Cicadellidae) and true bugs (Hemiptera) are seldom found in food products, and diagnostic features have not been worked out in this laboratory. Whole adults or nymphs, heads, or mangled insects, sometimes are encountered in leafy products. The bristle-like antenna and two ocelli on a pointed head which terminates in beak-like mouthparts, two rows of spines on the hind tibia, and the characteristic over-all shape, will usually suffice to identify the leafhoppers. (Fig. 6 B, C, D). The true bugs are characterized by the half-leathery, half-membranous forewings and the head prolonged into a beak (Fig. 6A).

2. Scale insects are markedly dissimilar from other types because of their size, presence of a shield-like scale, and degenerate appendages—except mouthparts which consist of highly specialized stylets. Leg fragments appear similar to those of many other groups of insects, as do body parts, although often the contamination consists of entire, shriveled, or mangled bodies (Fig. 4B). Small rudimentary insect fragments accompanied by some protective scales or fragments of scales, in the absence of positive findings that aphids or mites are present, usually will serve to confirm the presence of scale insects.

3. Aphid and thrips fragments are found together or separately, as isolated fragments, or in tremendously large numbers, in a wide variety of products, where they may be either serious economic pests or transient visitors caught in a food harvesting or processing operation. When aphid parts are present in any appreciable numbers, some of them will be the highly characteristic antenna (Fig. 7). The antenna consists of a setiform filament set on two slightly enlarged segments. The surface of the filament segments is covered with an irregular banding, or scale-like roughening,

[2] The information given in this discussion is intended to apply to contaminants of more than rare occurrence in food products.

and small circular sensori are scattered along its length. The last segment consists of a base and an abrupt narrowing into a terminal spur, terminating in sensorial hairs. The point of narrowing has several circular sensoria. Legs, too, are delicately elongate. The two-jointed tarsus has one pair of simple claws without any pad between them. All fragments are delicately clear.

Thrips too have a "landmark" structure; the name of the order "Thysanoptera" refers to the fringed wings which are highly characteristic, along with the barrel-segmented antennae and one- or two-jointed clawless tarsus, terminating in a membranous sac, which may appear to be an additional segment (see Fig. 8). It should be borne in mind that thrips—and also the other sucking insects mentioned above—do not have a true larval stage. In these cases, the immature forms resemble the adults except that they have no wings.

4. Some of the small, almost microscopic, springtails (Collembola) are field contaminants in a wide variety of products. They occur in various moist, usually dark situations, often where molds are abundant. Mushrooms may contain them, and they may be found drowned in sour and/or moldy cream stored in cool damp cellars. Because of their small size, whole insects may be found in the finished product. Descriptions of the group are given on Comstock (11), Essig (12) and other textbooks. The tarsi are one-jointed, and the abdomen bears a forked springing organ fastened on one of the posterior segments and recurved anteriorly under the abdomen.

5. In addition to the true insects infesting plants in the field, members of the class Arachnida may be found in a wide variety of products. Mites and spiders are the most commonly encountered contaminants, with pseudoscorpions (Part D) a poor third. In general external form, the mites and spiders are somewhat insect-like. However there are only one or two body regions, the young are not larvaform, there are no compound eyes, and there are four pairs of legs. Spiders are found everywhere feeding, for the most part, on insects, and hence may find their way into a variety of products. The mangled spider or cast skin usually can be recognized by the presence of fangs at the anterior end (Fig. 9) and the slender hairy legs, the segments of which articulate by means of elastic membranes rather than by the mono-, di-, or tri-condylic joints characteristic of most insect legs. Moreover, the spider legs and palpi are more regularly tapering, with joints less pronounced than those of the insects (Figs. 10, 11).

The mites may represent either field or storage contamination, the conditions under which members of this group live being as varied as all of the insects. Mites in a given food could come from an infestation of the growing succulent crop, from predatory mites parasitic upon an insect pest of the food, or from a direct storage infestation by mites. It is far

beyond the scope of this paper to discuss specific differences within this group.[3] Mites are minute arachnids with imperfectly developed body segmentation. Legs are simple, resembling elongate facsimilies of those from beetle larvae, and terminate in one or more claws. Although mite parts may be found in food products, and their separation from other thin-walled small fragments is quite difficult, the fact that the entire mite is quite small usually means that some whole, or practically whole, mites will be found in the product along with the fragments; and the diagnosis can be made on this basis. Of more significance is the problem of distinguishing mites from small ticks.

Although ticks do not infest food products, they have been found in them as contaminants.[4] The two groups, mites and ticks, are separated by taxonomists on the basis of the armament on the hypostome, and this character is the most satisfactory one to use in food contamination work. It is necessary to examine questionable specimens under the compound microscope (Fig. 12A).

B. *Field and Storage Contamination*

1. Ants may contaminate food in a variety of ways, being carried as contaminants on leafy vegetables, in raw materials such as cream or sugar, or as pests of the manufacturing establishment. Although their fragments more closely resemble the usual storage pests they are neither field nor storage. Isolated minute fragments are difficult to identify and, as is the case with other groups, identification is based upon the finding of some fragments bearing landmarks. The most commonly encountered characteristics are the smooth naked appearance of the cuticle, the antenna, the presence of a pedical, and the antenna brush on the forelegs (Fig. 13).

2. From the viewpoint of food contamination, the order Lepidoptera constitutes one of the most important insect groups. Altho the adults are significant only as egg-layers, larvae (caterpillars) infest a wide variety of products both in the field and in storage. A complete taxonomic treatment of food-infesting lepidopterous larvae is beyond the scope of this work, and we shall confine this discussion to the usual food-contaminants.[5] A primary distinction to be made is between caterpillars and their fragments, and beetle larvae and fragments (see Table 1). In making this separation—and in fact throughout all of this work—consideration must be given to the particular type of foodstuff involved, and to the common insect pests of the product. This is especially true when classifying the various caterpillar fragments, such as larva parts in corn.

[3] A recent study of this group is: The Mites Associated with Stored Food Products" by A. M. Hughes, Ministry of Agriculture and Fisheries, H. M. Stationery Office, London, England (1948) (13).

[4] Ticks have been found in churning cream, presumably having fallen into the cream during milking.

[5] Scattered thru the entomological literature are papers on various phases of this subject. These works, to a large extent, require the analyst to have a practically intact larva. For example see "A Comparison of Several Species of Lepidoptera Infesting Peach and Apple . . ." Maryland Agricultural Exp. Sta. Bull. 223, 1918; The Classification of Lepidopterous Larvae," Univ. Illinois Biol. Monograph, 2(1) (1915).

The corn earworm and several species of corn borers are the only common insect larvae found on ear corn, and hence are possible comminuted contaminants of canned corn. Smith (14) reported "In the corn earworm the frons extends only about half way up the head between the plates bearing the ocelli . . . , while in the corn borer it extends to the top of the head, completely separating these plates" (Fig. 14B, C).

TABLE 1.—*Differences between Lepidoptera and Coleoptera larvae*

LEPIDOPTERA	COLEOPTERA	ILLUSTRATION
Labium with short, horn-like projection anteriorly for spinning silk	Similar to those in adults	Figure 14
Pseudopods present	No pseudopods	Figures 15B
Cuticle (exclusive of head capsule) usually covered with a pattern of fine species or larger setae with pigmented basis	Cuticle (exclusive of head capsule) usually thin and translucent with scattered spines, and bases and entire cuticle all the same color	Figures 5, 15A, D, E
Posterior end terminates bluntly although with ventral anal pseudopod	Posterior end usually terminates in pigmented cerci or more or less rigid, horn-like elevations or protuberance	Figures 17A,B, 18
Usually more leathery white; pigmentation pink, green, grey, or black	Usually more horny, clear; when pigmented usually light amber	
Head capsule with adfrontal areas*	No adfrontal area	Figure 14B, C
Ocelli arranged in a distinct circle or crescent on each side of the head*	Ocelli variously arranged but not in large sweeping pattern of Lepidoptera	Figure 14A, B, C

* Nelson, R. W. (7).

Another characteristic structure is found in the skin of the body. A large part of the skin of the corn earworm is covered with very fine spines clearly visible at 40×. The skin of the borer is covered with very fine spots clearly visible as such at the same magnification (Figs. 5A, 15A).

A similar distinction can be made between corn earworm and tomato pinworm parts in tomato products. Howard (15) recognized that the small tomato pinworm was covered with a minute pigmented stippling, while the corn earworm cuticle was clothed with larger spines with a fine pigmentation at the base of each spine (Fig. 5A, C). Essig and Michel-

bacher (6) figure these characteristics, and also the shape and seta arrangement of the labrum, labium, palpi, spinneret, antennae, and mandibles of the corn earworm, tomato pinworm, and potato tuber moth (Fig. 14). Nelson (7) reports similarly and summarizes the chaetotaxy from the standpoint of insects commonly found in tomatoes.

Adult lepidoptera have a dense covering of scales; and whenever there are appreciable numbers of body parts present scales also will be present, so that the diagnosis of moths can be made from the scales, whether loose or clinging to the piece of cuticle (Fig. 15C).

C. *Storage Contamination*

1. The beetles, because of the large number of widely distributed storage pests in this order, contribute a high proportion of all of the insect parts found in cereals, spices, and other dried food products. Not only can pieces of adult beetles often be recognized as such, but many of the storage pests have characteristic parts which, even in a comminuted form, enable the analyst to make a specific determination. Adult beetle parts are relatively thick, usually amber to rust red, glistening, and with a regular surface pattern. By the presence of elytra fragments, or by mentally piecing the fragments together, the diagnosis of beetle contamination often is not too difficult. The larvae are distinguished from caterpillars by the structures given in Table 1.

2. Several species of grain-infesting beetles can be identified by their fragments. Fragments of the two *Tribolium* species, *confusum* and *ferrugineum*, can be determined by the forked anal cerci from the larva and the lateral combs on the pupa (Fig. 16). The sawteeth from sawtoothed grain beetle pupae and adults are useful landmarks, along with the simple truncated cone which terminates the posterior end of the larva and contains the anal opening. Larvae of cadelle, flat grain beetle, and dried fruit beetle (Figs 17 and 18), all have characteristic terminal appendages which may pass thru a food processing system with relatively little damage, and will serve to identify the specific contamination present. In addition, the cadelle has a prominent heavily pigmented prothoracic shield. The lateral combs mentioned above warrant special attention. They are present on most storage pests but the combs on each species are distinctly characteristic.

3. The weevils[6] and borers have larvae which are grub-like, legless (rice weevil, granary weevil) or with short, inconspicuous legs, and with a whitish integument that often fragments beyond recognition. The mouthparts contribute most of the weevil larva fragments and, since lar-

[6] The term "weevil" has been subjected to the vagaries of colloquial usage. Entomologically it specifically applies only to snouted beetles of the Superfamily Rhyncophora plus the pea weevils and bean weevils of the family Mylabridae (Bruchidae). The latter usually are not considered true weevils. The rice weevil (*Sitophilus oryza*) and granary weevil (*S. granarius*) are the only true weevils of any consequence infesting stored grains, other seeds, and caked cereals. They are not pests of comminuted cereals or milling equipment.

vae, pupae, and adults all feed in the same manner within the seed, adult parts are found along with those of the larva, and the diagnosis can be made on the basis of their fragments. The characteristic adult fragments are from the mouthparts, antennae, legs, and setae. The general shape of the body fragments also can be used (see Table 2). The finding of proboscis fragments, of course, is evidence of the presence of weevils. J. F. Nicholson, (19) reported the presence of club-shaped setae on some weevil parts

TABLE 2.—*Characteristics used in the separation of weevils and borers from flour beetles*

WEEVILS—BORERS	FLOUR BEETLES	ILLUSTRATION
Weevil mandible small, fan-shaped. Borer mandibles: Similar to flour beetle.	Mandibles of usual triangular shape; 2 prominent points of articulation.	Weevil: Fig. 19A Borer and Flour Beetle: Fig. 19B
Weevil antennae: with long basal segment, side insertion of the elbowed and club shaped flagellum. Borer antenna: large, curved basal segment followed by series of barrel-like segments, the last three abruptly enlarged into a serrate club.	Antenna of usual type with many small segments, each more distal segment fitted into, and somewhat larger than the next more proximal segment. Last segment pointed or rounded distally.	Weevil: Fig. 19C Borer: Fig. 19F Flour Beetle:[1]
Weevil tibia robustly rounded, terminating distally in pronounced points and with tarsus inserted into the side. Borer more of an intermediate type, terminating in rounded teeth and two sharp spurs. Terminal articulation with tarsus.	Tibia of "usual" type, enlarging distally, terminating in a few spines and with articulation to tarsus recessed into more or less squared-off end.	Weevil: Fig. 19E Borer: Fig. 19G Flour Beetle: Fig. 19D
Weevil with distally enlarged setae on various parts of the body. Borer: some similar setae on the elytral declivity. Some setae of the "usual" type.	Setae of usual type, enlarging rapidly at proximal end and gradually tapering to a point	Weevil: Fig. 15E Flour Beetle: Fig. 15D
Weevil: prothorax markedly pitted with round or elongate depressions. Borer: prothorax markedly and irregularly roughened.	Prothorax somewhat pitted with rather fine holes.	Borer: Fig. 15F

[1] For figures see Cotton (17); Linsley and Michelbacher (18), illustrations of sawtoothed grain beetle, flat grain beetle, confused flour beetle, rust red flour beetle, etc.

in grain, and during the course of routine examinations of a wide variety of cereal products it appeared that these setae, in distinction to the more elongate "usual" type, could be used to distinguish between fragments from weevils and borers infesting grain, and fragments from the "bran bugs" which may be found in grain but which are usually associated with cereal handling equipment or milled products. Concerning these setae L. L. Buchanan (20) commented as follows:

> "The distally enlarged setae on different parts of the body of the rice weevil and granary weevil, and the somewhat similar setae on the elytral declivity of *Rhizopertha dominica*, seem a reliable distinction between these 3 species and the following cereal and stored product beetles:—*Tribolium castaneum*, *Polarus ratzeburgi*, *Alphitobius diaperinus*, *Gnathocerus maxillosus*, *Tenebrio picipes*, *Tenebroides sp.*, *Cathartus quadricollis*, *Ahasversus advena*, *Oryzaephilus surinamensis*, *Caulophilus latinasus*, and *Araecerus fasciculatus*. Various other bettles (Curculionidae, Scolytidae, and some others) have similar setae, but none of these, so far as I know, are at all likely to be met with in cereal products."

D. *Miscellaneous Contaminants*

1. The flies are particularly repulsive and sometimes warrant special attention because of their filthy habits; breeding in fecal matter or decaying material; living promiscuously in both decomposing animal or vegetable material and in foodstuffs; and carrying pathogenic microorganisms. Adult flies and their eggs, maggots, and pupae may be found in a wide variety of foodstuffs. The eggs may be distinct and identifiable, as is the case with Drosophila (Fig. 20B), or similar to any number of other insect eggs. Dipterous larvae, or maggots, are nearly all legless, spindle shaped, and naked except for the presence of locomotive spines. The paired mouthhooks are the distinctive maggot fragment (Figs. 21 and 22). The deep pigmentation of these hook-like organs makes them readily apparent on a filter paper, and both the large shoulder-blade-like basal plate and protruding hooks have a form unlike the parts of any other insect. Adult fly parts usually are hairy. Although the peculiar knob-like halters (Fig. 20A) are positive proof of the presence of flies, they seldom are extracted from food products. Of more common occurrence is the diagnostic antenna bearing a plume like arista (Fig. 20A and 23). While fly antennae are not always shaped in this manner, this type of antenna is found only on flies, as is the fleshy proboscis ending in a spongy pad (Fig. 24A). The whole flies can be distinguished readily from the hymenoptera by the above characteristics, or by the fact that they have only two wings (one pair) while the hymenoptera have four wings (two pairs).

2. At times the examination of cereal products will disclose the presence of dark fragments with a metallic, blue lustre. These fragments do not resemble any of those from the common cereal infesting insects and usually

have numerous seta pits. Such fragments are from the small hymenoptera parasites of the primary grain infesting insects.

(3) Psocids (Fig. 24B) may occur in some cereal products. These extremely small insects may be present as minute, naked, translucent bodies with rather massive heads and robust femur segments of the leg; or the fragments, when present, usually will include some head or leg parts. The mandibles are comparatively large for the size of the insect.

(4) Another cereal contaminating insect is the silver fish. This insect is of relatively minor importance from the standpoint of numbers, damage done, and amount of contamination left in the product. However attention is called to the fact that this insect and the closely-related species, the fire brat, are covered with scales which bear a superficial resemblance to moth scales. However moth scales (Fig. 15C) terminate in large, crescent shaped angles, the apex of each crescent being at the end of each of the relatively few ribs of the scale. The distal end terminates in abrupt angles. In contrast, the silverfish and fire brat scales have numerous ribs, each terminating at the apex of a minute tooth, the complete serrate edge being gradually rounded distally (Fig. 24C). In fact, the overall shape of a moth scale is that of a wedge, while that of the two scale-bearing Thysanura is ovate-elongate to heart-shaped.

5. Figure 24E shows the claw from a pseudoscorpion. These minute arachnids are found under bark and in numerous other secluded places, and therefore may be found in cinnamon, pepper, and other spices. In finely ground material the chelate palpus, or claw, is the characteristic fragment.

6. The insect trachea and tracheoles, with their spiral thickenings, (Fig. 24E) are the only soft internal organs which are extracted from the food and determined as insect material, and even this structure is readily determined only when some of the larger caterpillars are involved.

V. CHEMICAL TESTS, STAINS, AND OTHER DIAGNOSTIC FEATURES

While the diagnosis of various insect, or suspected insect, contaminations is usually based on their morphology, a study of the problem would not be complete without at least mentioning some of the work on chemical tests and stains. The classic test for chitin is that of Campbell (21) who analysed for the decomposition product chitosan. This test is not adaptable to routine fragment count analyses. Several stains have been used on chitin, but the recent work by Gier, Wilbur, and Miller (22) is the only published material on a staining procedure for use in connection with fragment count analyses. The work on cellulose reactions has been reported in various publications. Hanausek (23) summarizes many of the tests suggested up to that time. These tests with chlorozinc iodide and cuprammonia, and the tests for lignin, are still widely employed.

Mention also should be made of certain differences between insect parts contributed by insects living in a product, and parts produced by the grinding of the insects in the preparation of a comminuted food product. Insect parts from what may be termed "normal wear and tear" consist mainly of appendage fragments: leg parts, antennae, palpi, wing covers, and mandibles. When the parts come from the use of an infested raw material in the preparation of a comminuted product, the appendage fragments will be no more numerous than would be expected from their relative numbers on the insect itself. In either case, larval parts will consist mainly of pieces of the head which are more readily located because of their pigmentation, and recognized because of the greater abundance of landmarks on the head. In fact some pieces of the head contribute a substantial number of the insect parts found in cereal products. The labrum (Fig. 24D) and the clypeus to which it is attached, and the gena, or cheek, including the point which articulates with the mandible (Fig. 3B), all warrant special attention since they break into numerous pieces that lack the eye-arresting pigmentation that is present in the mandible.

VI. CONCLUSION

The continuation of this work can come from many fields of investigation, using both new and old tools at our disposal. The insect taxonomist may be able to apply his vast fund of information to this relatively new field. The chemist may simplify existing tests, or develop new ones that can be used for routine examinations. Already the entomologist and biologist have joined forces to give us a promising stain for insect fragment differentiation (22). The phase microscope awaits our use, and inexpensive polarizing systems have been used on wide field microscopes in this laboratory. The future holds much promise, and it is hoped that this paper will be of some value as a guide along the way.

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Figs. 1 to 25 appear on the following pages.

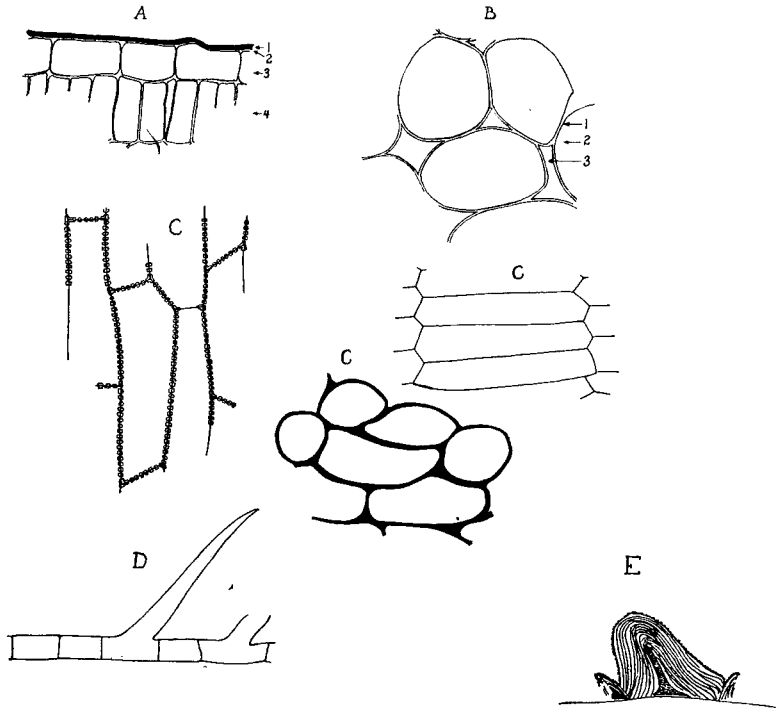


FIG. 1. Plant cell patterns and structures. A. Cross section of leaf epidermis showing (1) non-cellular cuticle which is not visible except in especially prepared sections, (2) outer wall of epidermal cells, (3) epidermal cells, (4) leaf palisade parenchyma. B. Parenchyma tissue showing (1) cell wall, (2) cell cavity (3) intercellular space. C. Cell patterns from wheat bran. D. Diagram of plant hair arising as an outgrowth of epidermal cell. See also multicellular plant hair in Fig. 12, C. E. Outer epidermis of corn cockle seed showing tubercle in cross section.

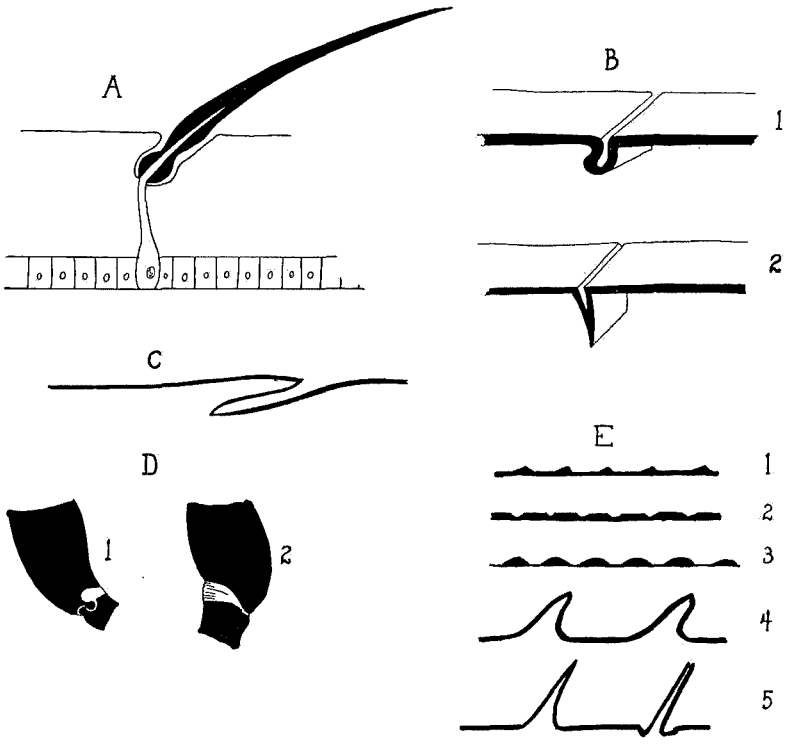


FIG. 2.—Insect cuticle. A. Diagrammatic cross-section showing seta embedded in the clear cuticle (cells visible only after special staining and treatment). B. Schematic representation of two types of insect sutures. C. Simple infolding of cuticle along suture, shown in cross section. D. Leg Joints: (1) ball and socket; (2) elastic membrane and pivot. (Black = rigid cuticle; clear = membrane.) E. Types of insect external processes in cross section: (1) Simple nodules or ridges; (2) Pits; (3) Roughened pattern on otherwise thin cuticle; (4) Fixed spines caused by hollow elevations of the cuticle; (5) Movable hairs, or setae.

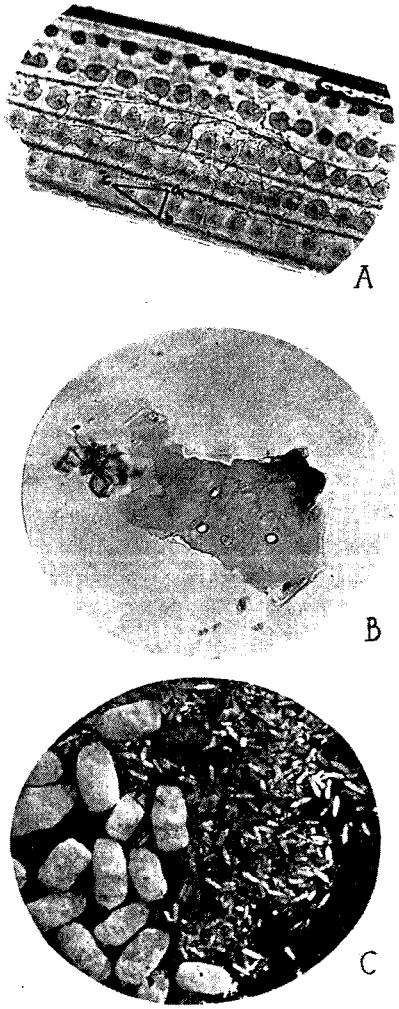


FIG. 3.—A. Part of elytra from confused flour beetle; a-b-c indicating fragment that could be diagnosed by surface pattern. B. Part of "cheek" from flour beetle larva showing translucence, empty seta pits, and rigid pigmented point of articulation to mandible. C. Two types of insect excreta. Smaller from flour beetle. Larger from caterpillar infesting stored food product.

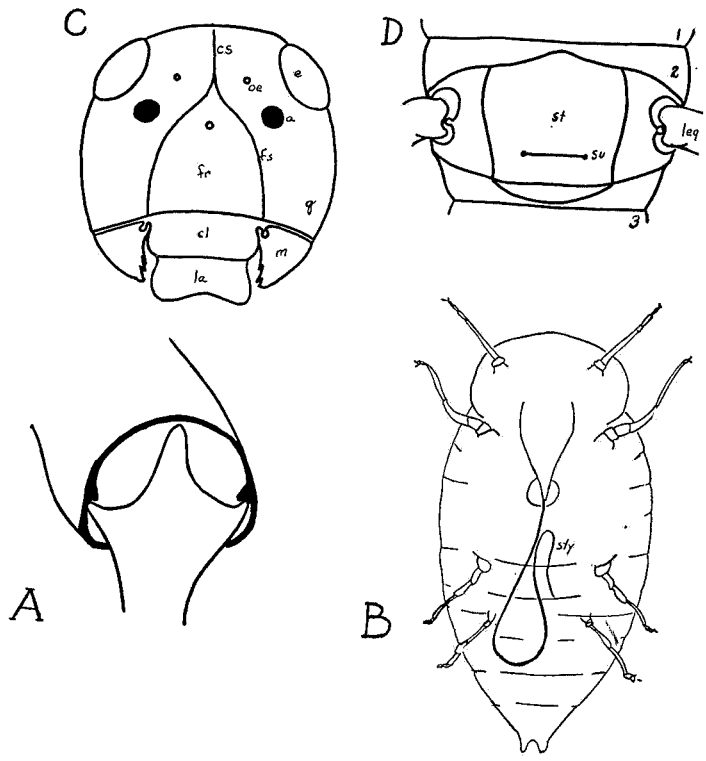


FIG. 4.—Some details of insect structure. A. Leg joint showing pivots and lever; B. Highly degenerate scale insect; C. Grasshopper head showing main sutures; D. Venter of thoracic segment showing some of the sclerites; a. antenna, cl. clypeus, cs. cranial suture, e. eye, fr. frons, fs. frontal suture, g. gena, la. labrum, m. mandible, oc. ocellus, st. sterum, sty. sucking stylets, su. suture; 1, 2, 3, respectively, pro-, meso-, metathorax.

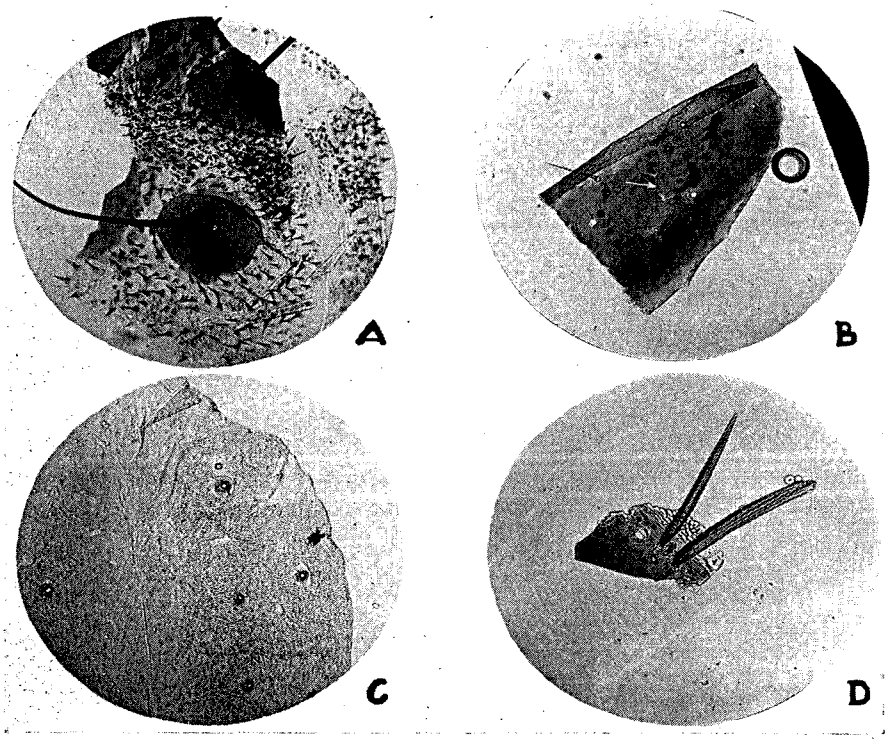


FIG. 5.—A. Corn earworm skin fragment showing surface spines, setae and seta bases; B. larva fragment showing translucence, setae and bases and irregular crack induced by probing; C. Pinworm skin showing very fine surface spines and seta bases; D. Insect fragment from cornmeal showing translucent portion; junction of translucent and roughened skin, also ball and socket base on setae. A and C by B. J. Howard.

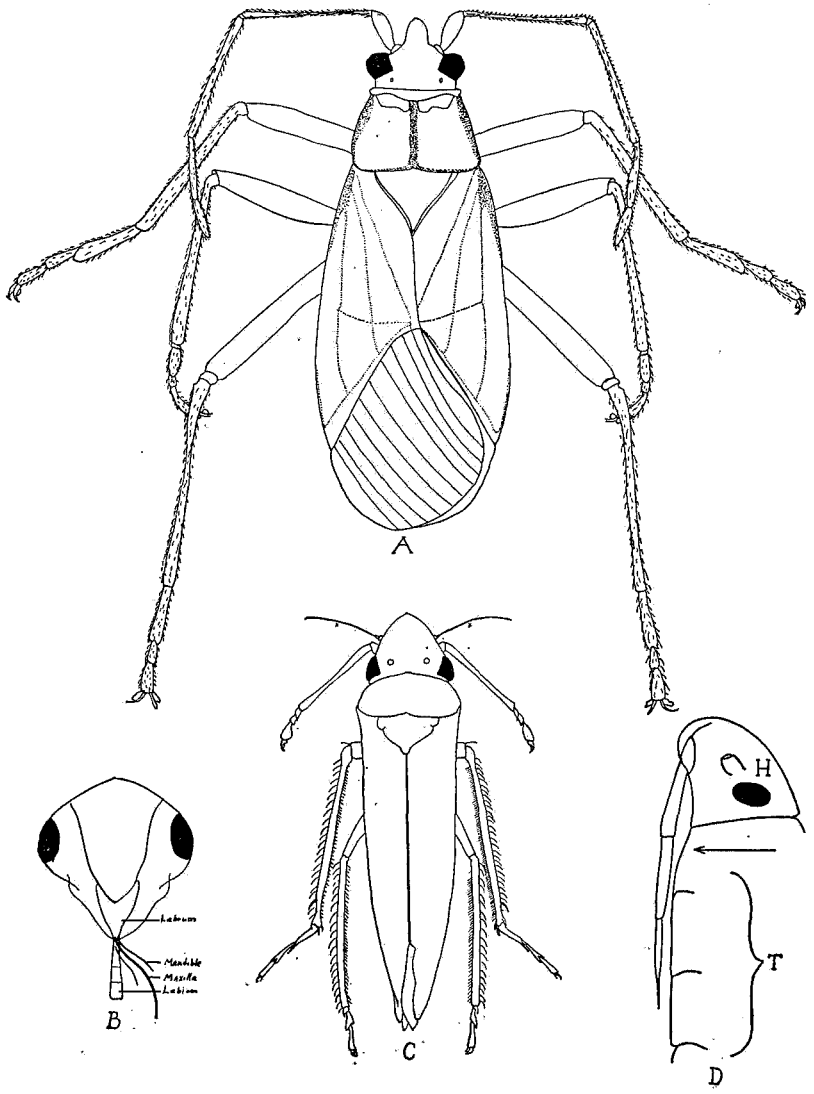


FIG. 6.—Bugs and Leafhoppers. A. Dorsal view of true bug; B. Anterior view leafhopper head showing sucking mouthparts; C. Dorsal view of leafhopper; D. Lateral view of leafhopper or bug showing elongate, posterior-ventrally extended proboscis (H. T., head and thorax; arrow points ventrally).

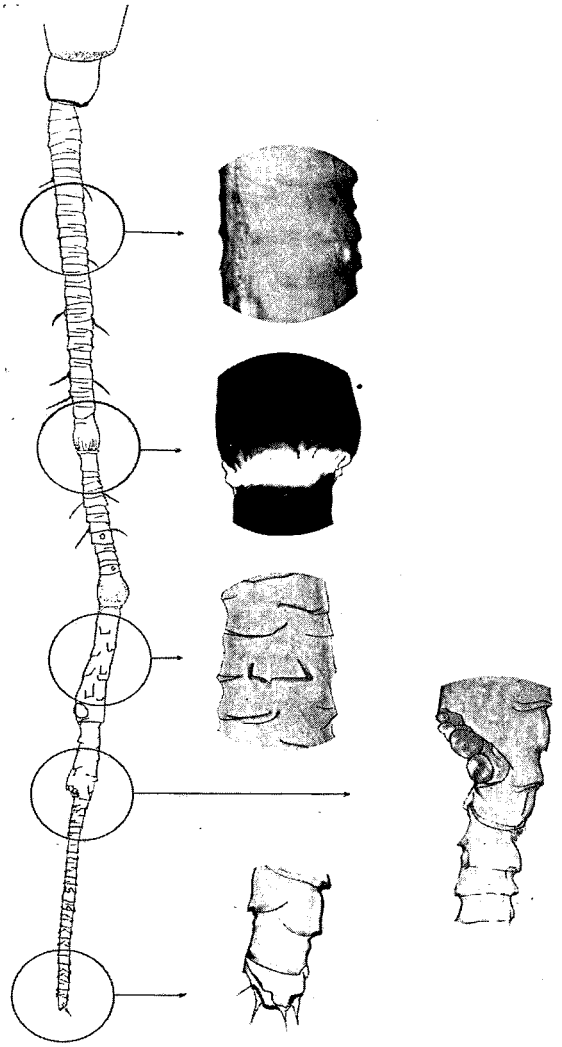


FIG. 7.—Aphid antenna. Inserts show detail of surface pattern.

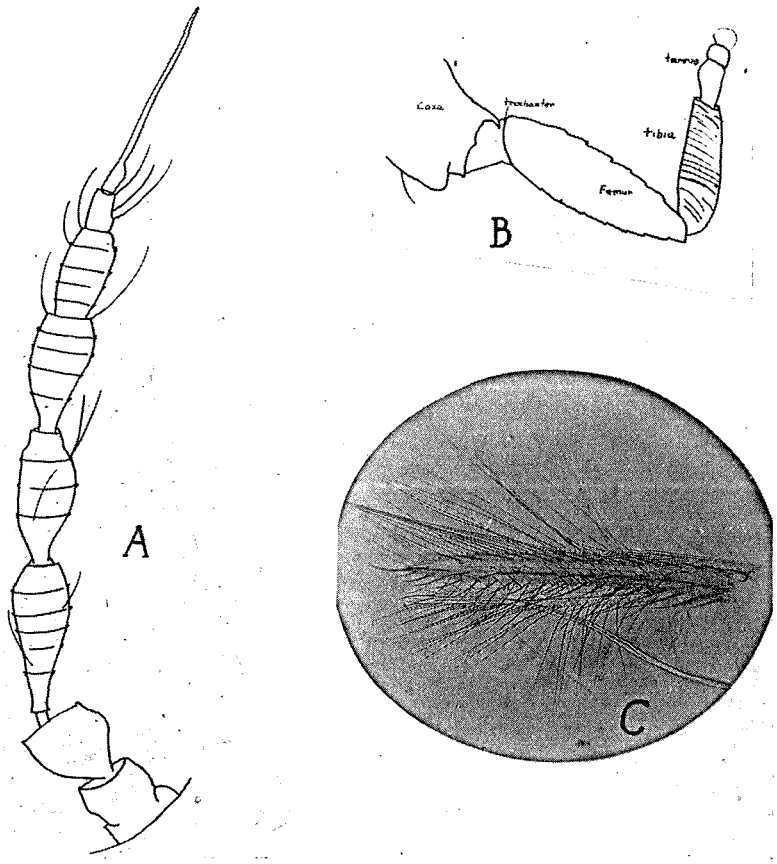


FIG. 8.—Thrips fragments. A. Antenna; B. Leg; C. Wing. Not drawn to same scale.

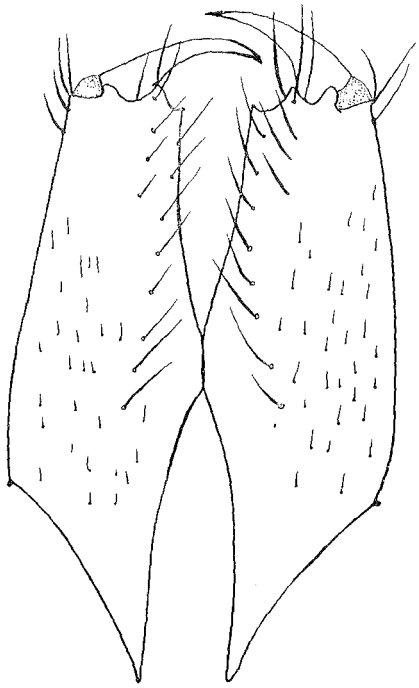


FIG. 9.—Spider poison fangs (stippling: membrane).

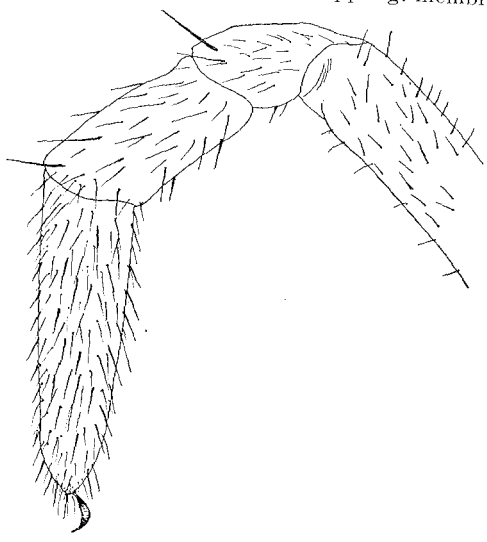


FIG. 10.—Spider palpus.

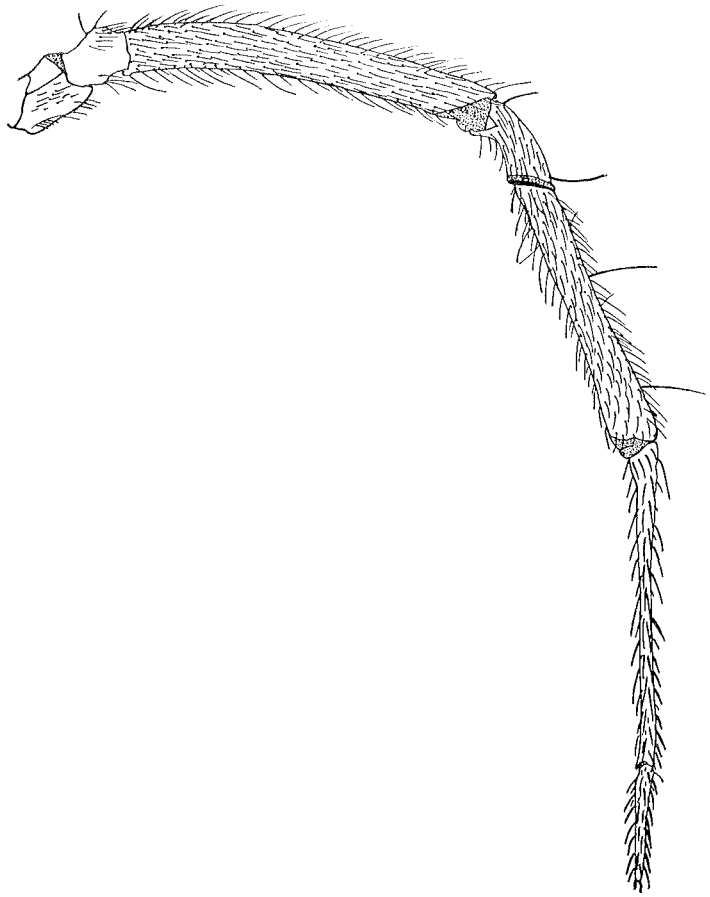


FIG. 11.—Spider leg (stippling: membrane).

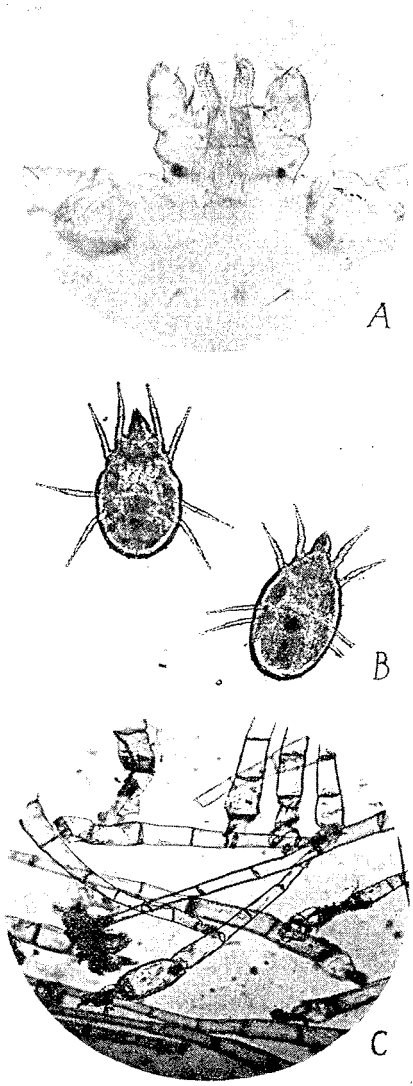


FIG. 12.—Mites. A. Toothed hypostome of small tick. Photograph by F. R. Smith (Food & Drug Adm.) from material of E. W. Baker (Bur. Entomology and Plant Quarantine). B. A cereal mite. C. Multicellular plant hairs. Cell walls resembling leg segments but not true joints. A and C much enlarged in respect to others in this figure.

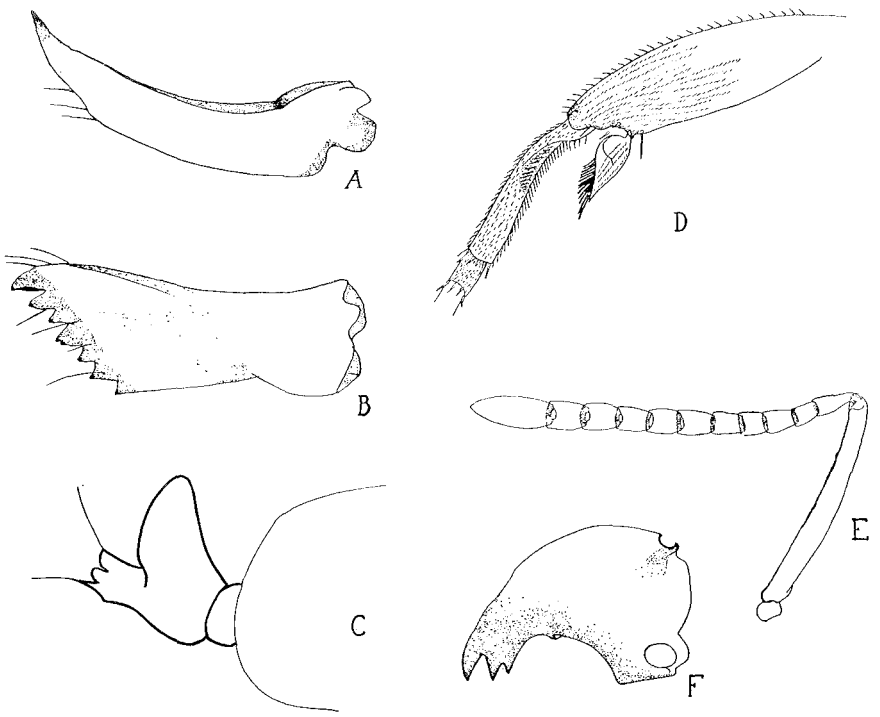


FIG. 13.—Ant parts. A. Anterior aspect of ant mandible showing scythe shape; B. Ventral aspect of same mandible showing spade shape; C. Schematic cross section of pedical between thorax and abdomen; D. Foreleg showing antenna cleaner at the tibia-tarsus joint consisting of a comb-like "thumb" with groove on the tarsus; E. Antenna showing greatly elongate first joint, or scape, and remaining shorter joints, or flagellum, articulated at an angle to the scape; entire antenna slender, tapering, individual joints not separately nodular as in some beetles. F. Sickle-shaped type of ant mandible.

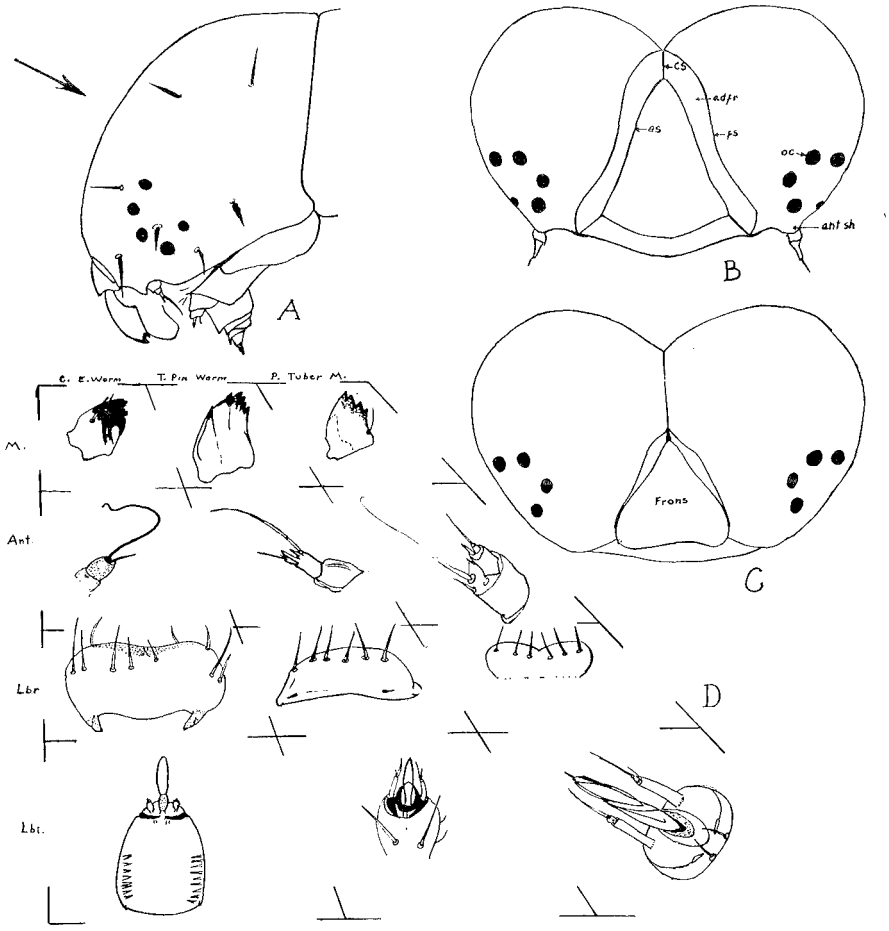


FIG. 14.—Caterpillar head parts. A. Side view head. Arrow indicates angle from which B & C drawn. B. Corn borer head showing epicranial suture (es) and frontal suture (fs) extending up almost to vertex of head. Coronal suture (cs) short. Antenna shield (ant sh) projecting over antenna. Ocellus (oc). Adfrontal area (adfr). C. Corn earworm head with long coronal suture. D. Corn earworm, tomato pinworm, potato tuber moth, diagnostic head parts. Mandible (M), Antenna (Ant), Labrum (Lbr), Labium with spinnerete (Lbi), Antenna shield after Capps (16). Figures in D taken from Essig and Michelbacher (6).

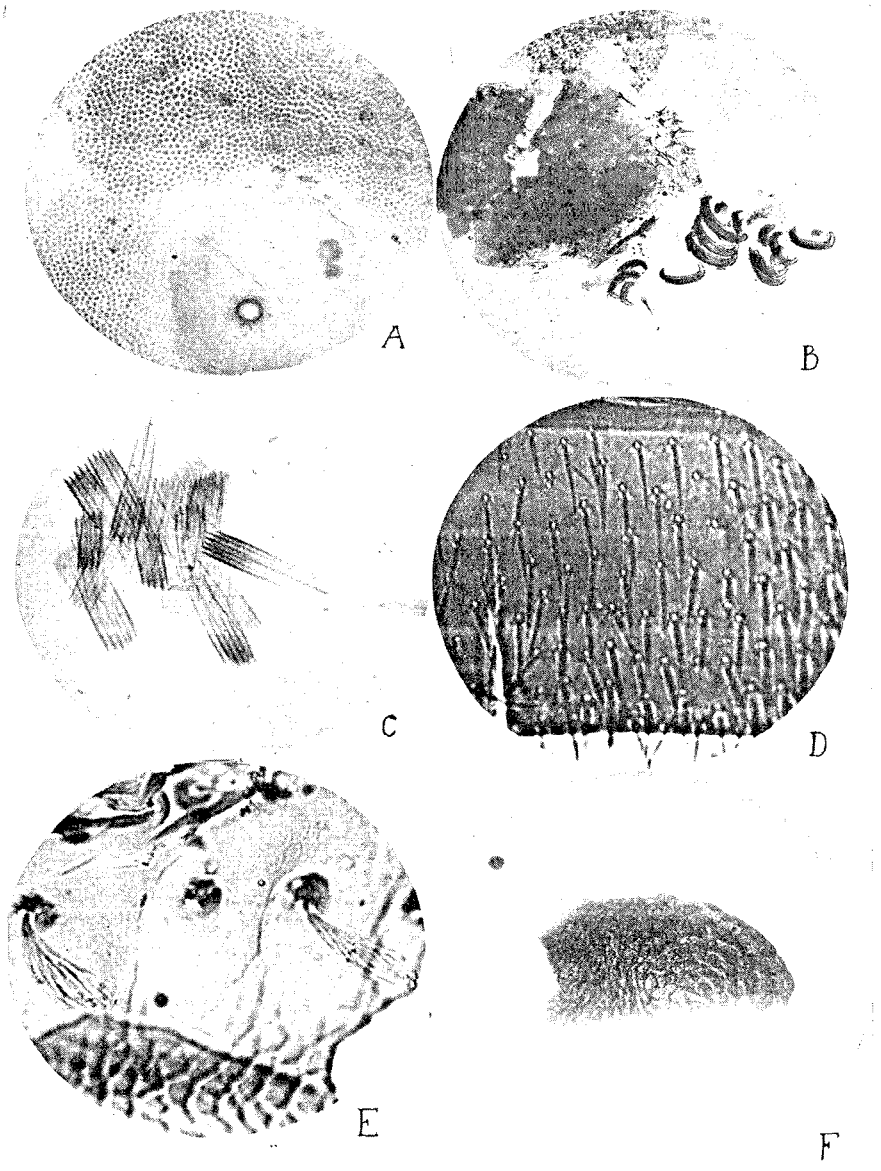


FIG. 15.—Caterpillar and Beetle Parts. A. Corn borer skin; B. Mangled pseudopod as encountered in comminuted products; C. Moth scales; D. Flour beetle setae; E. Grain weevil setae; F. Weevil thorax part. (A. by F. R. Smith; B. by B. J. Howard; D. and E. by J. F. Nicholson. See also Fig. 5A Corn earworm skin and 5 C Tomato pinworm skin.)

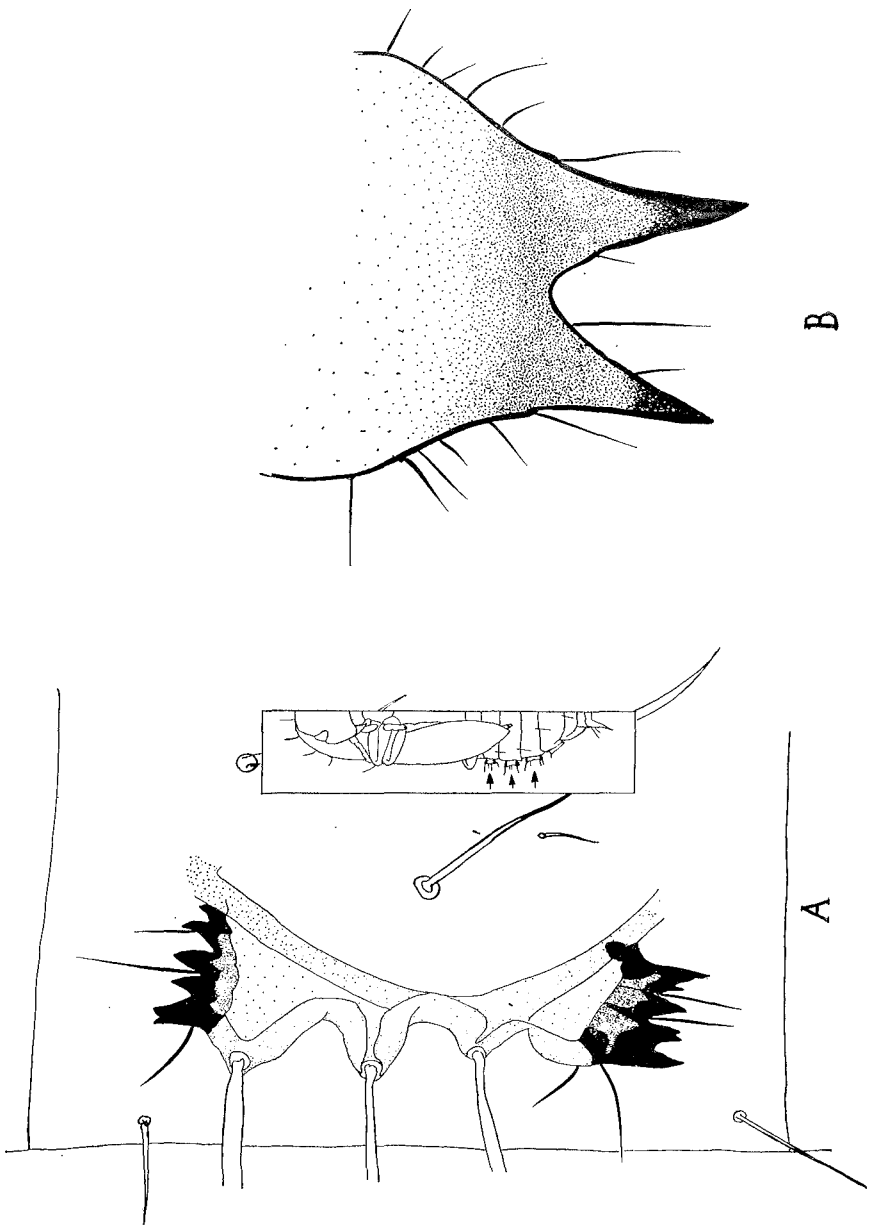


FIG. 16.—Tribolium pupa and larva, characteristic fragments. A. Lateral combs on pupa; insert shows location of combs on ae pupa. B. Anal cerci from larva. (Not drawn to same scale).

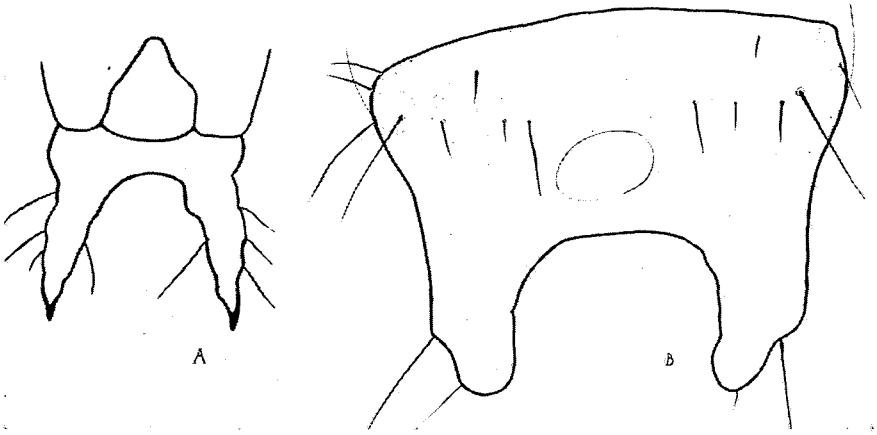


FIG. 17.—Anal cerci: A. Flat grain beetle; B. Cadelle. (Not drawn to same scale.)

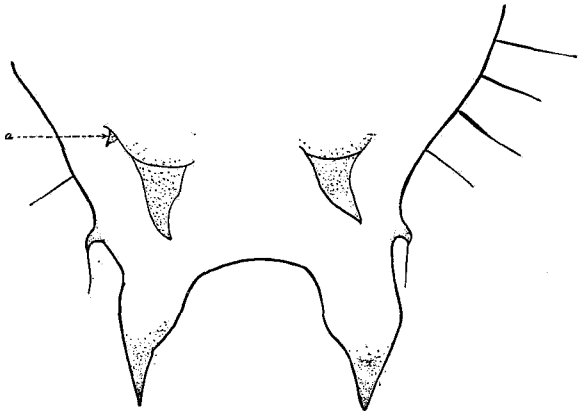


FIG. 18.—Anal cerci of dried fruit beetle. Supernumerary cerci such as "a", paired or unpaired, may be present.

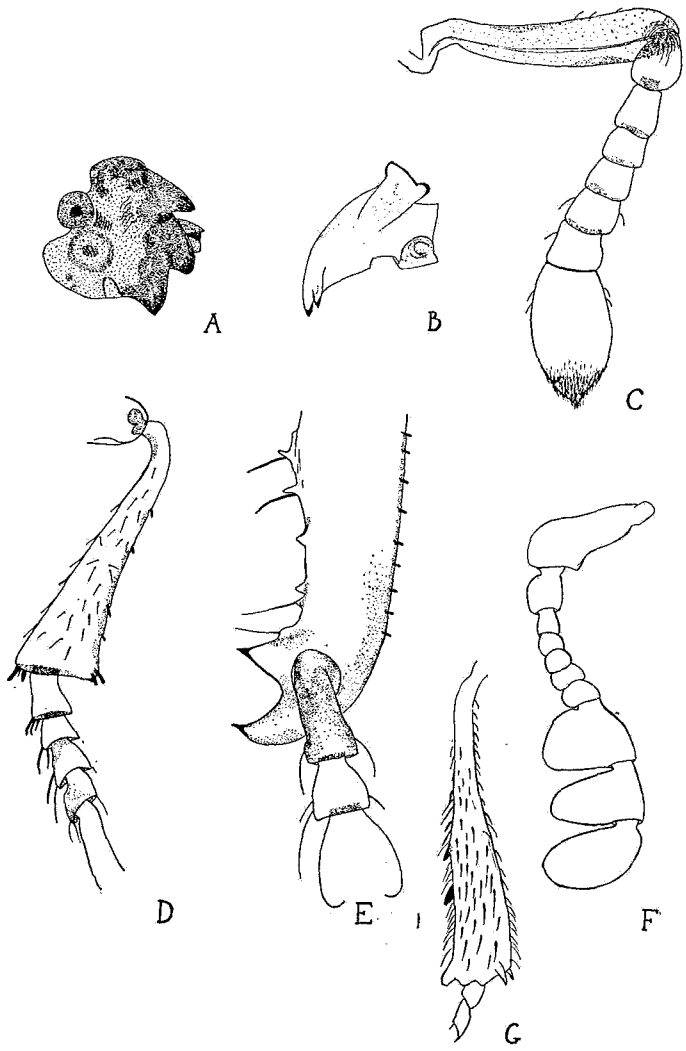
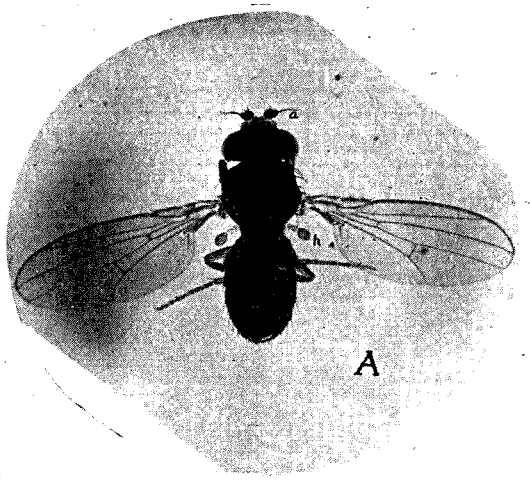


FIG. 19.—Weevil and borer vs. beetle parts. A. Weevil mandible; B. Confused flour beetle mandible; C. Weevil antenna; D. Confused flour beetle tibia and part of tarsus; E. Weevil (same as D); F. Lesser grain borer antenna (hairs omitted); G. Lesser grain borer leg (as in D.)



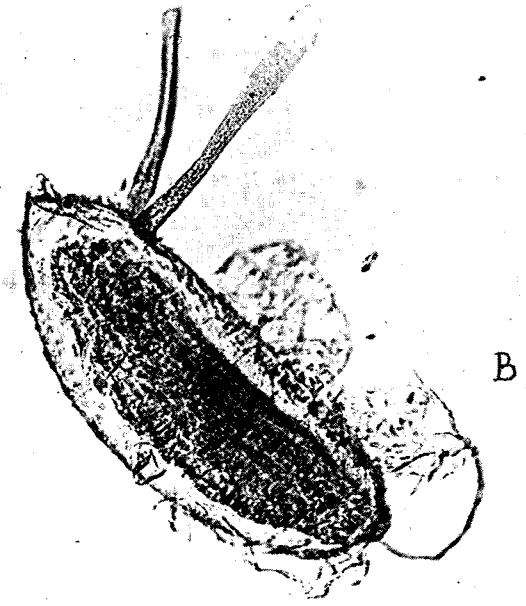


FIG. 20.—A. Dorsophila adult; (h) halter; (a) arista on antenna B. Drosophila egg, greatly enlarged in respect to the adult, showing characteristic prongs. Surface reticulation does not appear in the photograph.

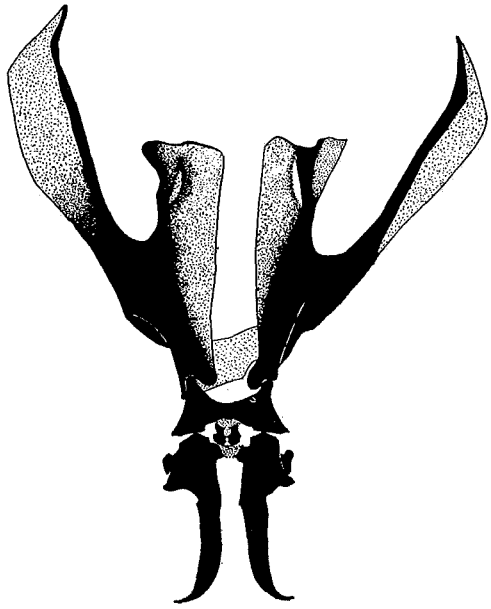


FIG. 21.—Maggot mouthhooks. Dorsal view.

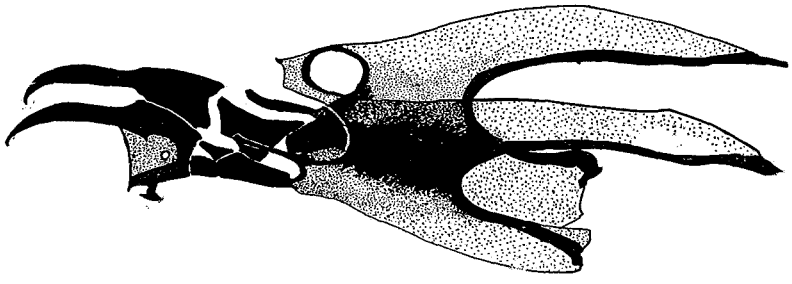


FIG. 22.—Maggot mouthhooks. Lateral ventral view.

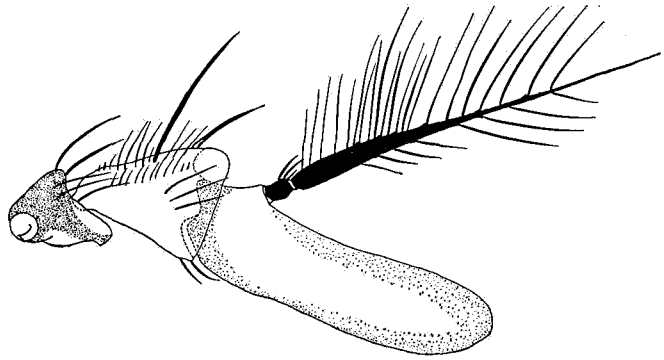


FIG. 23.—Fly antenna with arista.

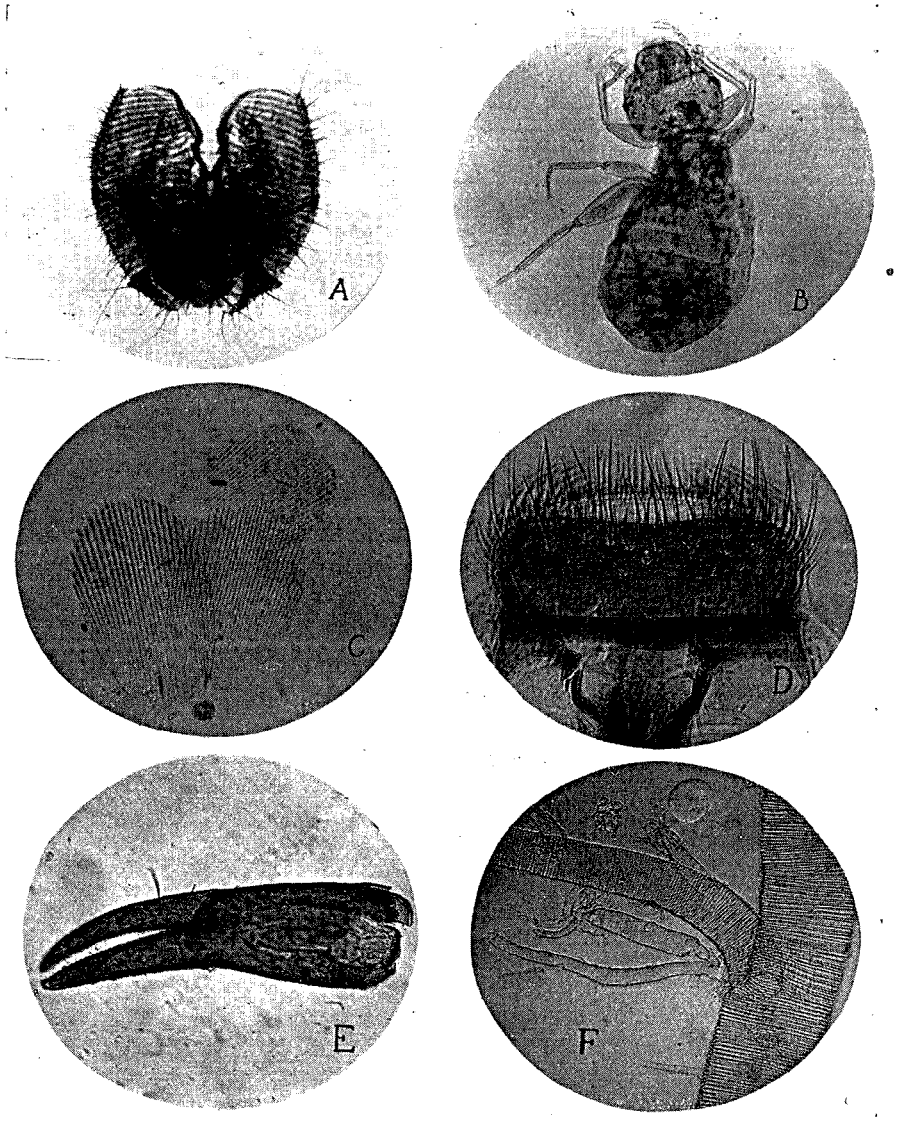


FIG. 24.—Insect parts and a Psocid. A. Terminal pad of fly proboscis; B. Psocid; C. Silverfish scales; D. Flour beetle labrum; E. Chelate palpus from pseudoscorpion; F. Corn earworm trachea.

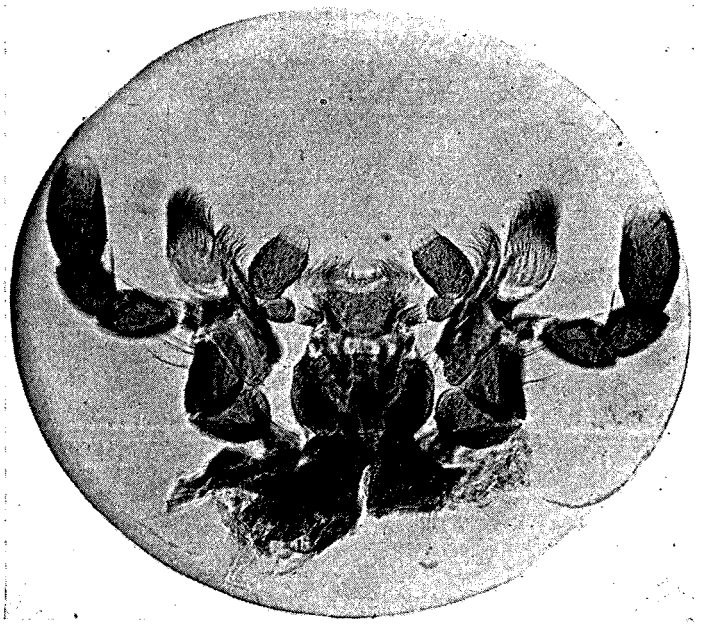


FIG. 25.—Tribolium labium, labial palpi, maxilla, and maxillary palpi to show symmetry and complexity of insect parts.

NOTE

A NEW SEPARATORY FUNNEL FOR ETHER EXTRACTIONS

By J. H. CANNON (Food and Drug Administration, Federal Security Agency, St. Louis, Mo.)

The separatory funnel here described offers a simple way of making ether extractions as easy as chloroform extractions. The advantage over the ordinary separatory funnel is that no transfers are required during the extraction. The advantage over the Mojonnier tube is that much sharper separations are possible, and the volume of aqueous phase is not so critical.

The device can be easily constructed in the laboratory from standard pyrex tubing, stopcocks, and ground joints obtainable from supply houses.

To use, open stopcock "A" and remove stopper-stopcock assemply "B." Introduce the sample into limb 1 and add water (acid or alkali solution if necessary) in sufficient volume to fill limb 2 and the curved portion connecting 1 and 2. (So that the curved portion will still be filled with aqueous phase after the ether extract has been forced out through the capillary.) Allow effervescence of other reaction to subside. Add a suitable volume of ether, replace stopper assembly "B," and secure with rubber bands or springs.

Close both stopcocks and agitate, either by shaking or by inverting repeatedly. Allow the phases to separate, and bring all of the aqueous phase into limb 1 by suitable manipulation. Open stopcock "A" to relieve pressure. Slip a rubber tube on the inlet of stopcock "B." Open stopcock "B." Blow gently through the rubber tube and manipulate stopcock "A" to allow the ether extract to flow out through the capillary outlet into a tared beaker or other receiving vessel.

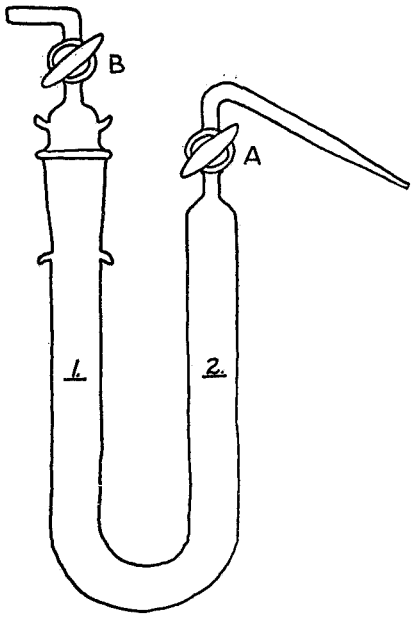


FIG. 1.—Separatory funnel for ether extraction.

The Colloid Chemistry of the Silicate Minerals. Vol. 1. of Agronomy, A Series of Monographs prepared under the auspices of the American Society of Agronomy. By C. Edmund Marshall, Professor of Soils, University of Missouri, Columbia, Missouri. Academic Press, Inc., New York, N. Y., 1944. ix+195 pp., 85 illus. Price $5.80.

There has been a need for a book on this comparatively new, yet complicated, subject. Its importance to agriculture warrants its selection as the first monograph of a series on agronomic topics. The author's long experience in various phases of silicate chemistry makes him a proper choice for the preparation of a review work on this subject. The book may be adjudged as relatively small, but its size results not from a narrow breadth of material, but from Dr. Marshall's abilities in clear, concise writing.

In general, a conscious restriction limits the presentation to reasonably pure materials, because the goal of this publication was the clarification of fundamental aspects. For this reason much of the chemical work on soils is not considered. Some space is devoted to three-dimensional framework silicates, especially zeolites, but the main attention is centered on the silicates with planar frameworks, which represent the important clay minerals of soils. Little or no space is wasted on details of methods or techniques, and results and principles occupy the center of the stage throughout.

The structure and composition of the various clay minerals, together with their sizes and shapes, are discussed in detail in several chapters. This reviewer noted with interest that in the chapter on ionic exchange, Dr. Marshall regards the different cation replacement series found for various clays by Schachtschabel, not as a valid criticism of the hydration theory, but rather a result of drying ammonium-saturated clays prior to the replacement studies. Principles and results of investigations by the author and coworkers on clay membrane electrodes are discussed in chapters on properties of clay films, and on the titration of clay acids. At the present time the general validity of measurements of pH and cation activities of colloidal systems, is under attack on the basis of cation replacement by the potassium of the KCl of calomel electrode salt bridges. Other chapters discuss optical properties, adsorption, and electrokinetic and mechanical properties.

Very few technical errors were noticed. However, several of these observed in certain of the figures warrant calling to attention. In Figure 67, the second curve from the top should be labelled "NaCl," not "NaOH." in Figure 68, the "a" of $CaCl_2$ should be lower case. In Figures 70 and 71, the word "log" does not belong in the abscissa legend. In Figure 72, the value "90" on the abscissa scale should be "80;" in the abscissa legend "100" should be inserted before "gm." Figure 74 on page 156 is not referred to until page 163.

References are grouped at the end of each chapter, while general author and subject indices are in the rear of the book. The binding is satisfactory, the paper of good quality, and the type and illustrations legible. This monograph is recommended for all soil scientists and colloid chemists. R. F. REITEMEIER

Advances in Agronomy, Vol. 1. Edited by A. G. Norman, prepared under the Auspices of the American Society of Agronomy, Academic Press, Inc., New York, N. Y. (1949). xii+439 pp. Price $7.50.

The enormous subject matter included in the field of Agronomy is well illustrated by the diverse chapter titles that follow: Plant Growth on Saline and Alkali Soils; New Fertilizers and Fertilizer Practices; Soybeans; The Clay Minerals in Soils; Alfalfa Improvement; Soil Micro-organisms and Plant Roots; Weed Control; Boron in Soils and Crops; Potato Production; and Fixation of Soil Phosphorus.

Definitely chemical subject matter is discussed in the chapters dealing with clay minerals and fixation of phosphorus. "Plant Growth on Saline and Alkali Soils" is a

splendid treatise involving chemistry and plant physiology. The chapter entitled "Soil Microorganisms and Plant Roots" is definitely in the field of biology. The longest chapter, entitled "Soybeans," is essentially a monograph on the subject. It summarizes up-to-date information on many phases of the breeding and culture of this important crop; in fact, most of the information one might want on this subject is there. The same may be said of the chapter on Alfalfa Improvement. Insect relations are extensively discussed here.

The bibliography of each chapter is extensive and appears to have been well chosen. The chapter entitled "Boron in Soils and Crops" covers the subject splendidly. It would seem, however, that the title and content of the chapter might well have been expanded to include a few of the other minor elements such as copper, zinc, and manganese, even though boron holds a leading place among minor elements. It is true, however, that some mention is made of these minor elements in the chapter concerned with potato production.

Many agronomists will want this excellent book in their personal libraries.

M. S. ANDERSON

Agricultural Chemistry. A Reference Text. Editor-in-Chief, Donald E. H. Frear, 812 pages, D. Van Nostrand Co., Inc., New York. Price $9.00.

The editor presents two volumes on the more important phases of agricultural chemistry. In this, the first, its twenty-four chapters are contributed by twenty specialists in their respective fields, eighteen of whom are on the faculties of colleges and universities. Volume I deals with the chemistry of the basic compounds of biological importance, and the physiological processes of plants and animals. It is designed to supply knowledge on the chemistry of agriculture, and thus to be most useful to the teacher and research worker. As a research text, it is primarily designed to serve the graduate student and specialists engaged in agricultural work, but it should also be of value and interest to those in industry engaged in development promotion, sale, or application of the products of agriculture. Vol. II will deal with practical applications of agricultural chemistry.

The first three chapters, comprising Part I, deal with the chemistry of the fundamental materials of the living organism: carbohydrates, lipids, and proteins. Each chapter discusses their structure, properties, reactions, separation, identification and classification.

Part II is devoted to chemical processes of fundamental importance in agriculture. One chapter deals with enzymes, their classification and the nature of their catalytic actions. The other chapters cover biochemical oxidation reactions, biophysical phenomena and cell chemistry.

Part III is devoted to plant chemistry with chapters dealing with photosynthesis, plant pigments, hormones, utilization and absorption of inorganic substances, carbohydrates and nitrogen compounds.

Metabolism of the animal is the fundamental subject of the several chapters of Part IV on animal chemistry in its various aspects, such as energy, carbohydrates lipids, and protein. The final chapters of Part IV bear on digestive processes, and absorption and utilization of inorganic substances and vitamins.

No treatise on the chemistry of agriculture can be complete without a discussion of fundamentals of soil chemistry. Thus the volume closes with three chapters devoted to the minerals, colloids and organic matter of soils. Although no special chapter is devoted to the use of radioactive isotopes, there are several brief discussions of the application of these newer tools of research in Part III on plants.

Throughout the book one finds discussions of principles of methods of analysis, but details of procedure are not given; however references to sources of analytical directions are included in the bibliographies. Each chapter is supported by an extensive bibliography, which in the case of vitamins includes 394 references.

HENRY A. LEPPER